# . A STUDY OF THE METABOLISM OF THE HERBICIDE 3,6-DICHLOROPICOLINIC ACID BY A STABLE MIXED MICROBIAL POPULATION ISOLATED FROM SOIL

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy.

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DECLARATION

I declare that this thesis is a report of research undertaken by myself during the years 1976-1979 in the Department of Environmental Sciences under the supervision of Dr. J.H. Slater. It is my own original unaided work and to the best of my knowledge has not been previously described by any other persons.

## SUMMARY

A stable mixed microbial community able to utilise picolinic acid as the sole carbon and energy source was isolated from soil by continuous-flow culture enrichment. The community contained six different organisms three of which were capable of growth on picolinate in pure culture. These three primary utilisers were Pseudomonas aeruginosa, Alcaligenes faecalis and a second Alcaligenes sp. The three secondary organisms were Bacillus licheniformis, a Rhodococcus sp and a Corynebacterium sp. of the C.aquaticum type. These secondary organisms were presumed to be growing on the secondary metabolites of the primary utilisers but contributing something to the mixed culture due to its stability.

The community was adapted to degrade a chlorinated analogue of picolinate, namely 3,6-dichloropicolinate, and the kinetics of enzyme attack on picolinate, in the presence and absence of the chlorinated compound was studied.

At low growth rates, that would represent nature more accurately, there was a decrease in 36-DCPA concentration of approximately 15% indicating some microbial degradation. Oxygen uptake by the community in the presence of various substrates was studied and, generally, it was found that 36DCPA inhibited, to some extent. the uptake by the microbial community.

CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 The Need for Pesticides

The loss of food due to the action of pests has been placed as high as 50% in developing countries, and it has been estimated that the total loss of food production could supply 300 million people with 2,500 calories a day every year (Snelson, 1977). If man is unwilling, or unable to limit his own population then it is essential that a means of minimising the loss of agricultural produce is found.

In more scientifically advanced countries there is continual breeding of plant species to try to establish a variety of organism that has a higher yield per acre than either parent strain. This is achieved by much inbreeding of plant species and generally results in a less hardy variety of plant. Thus the high producer tends to be more susceptible to disease. In order to maintain the high production of food required, it is necessary, therefore, to reduce the population of disease producing organisms.

To cope with the problem of providing enough food for an ever increasing population pesticides have been developed for every stage in the production of food. Initially, selective herbicides, insecticides and fungicides are applied to the field to reduce competition and disease. After the harvest more fungicides are added to prevent deterioration of the crop. Clearly, then, there is a need for pesticides, but this need must be measured against the deterioration of the environment due to the introduction of recalcitrant compounds.

It is also clear that there is some need for control and testing of compounds being released into the environment. There is a need to test the pesticide against any non-target species of animal or plant that might be exposed to the herbicide. It is especially important to test the effect of the herbicide on non-target microorganisms since they are important agents in the maintenance of soil fertility.

## 1.2 Secondary Effects of Pesticides on Soil Microorganisms

Although it is relatively difficult to assess the secondary effects of pesticides on soil microorganisms *in situ*, it is a more intelligible study when performed in the laboratory. Greaves (1979) studied the long term effects of herbicides on soil microorganisms and concluded that there was little evidence of direct effects of herbicides on microorganisms or their activity in the soil. Other workers have found similar results (Kaiser, Pochon & Cassini, 1970; Horowitz, Blumenfeld, Herzlinger & Huling, 1974). However, some herbicides were found to depress nitrification in the soil (Chandra, 1964) while others inhibited carbon dioxide evolution (Chandra, Furtick & Bollen, 1960). Lewis, Papvizas & Hora (1977) studied the effects of herbicides on microbial activity in the soil in amounts comparable to field application rate and suggested that at these rates the 25 herbicides tested did not affect the overall microbial activity of the soil microflora. Roslycky (1976) found similar results but did observe that oxygen uptake was decreased to some extent by certain herbicides.

## 1.3 The Disappearance of Pesticides from the Environment

The introduction of novel pesticides for selective control of some species is dependent not only on its pesticidal activity but also on its eventual fate. A pesticide should destroy the target organism and then dissipate to prevent an accumulation in the soil or the harvested crop. In this context dissipation must not be interpreted solely as removal from the global environment but by degradation or decomposition to mineral constituents.

In order for the degradation of the pesticide to occur there must be a microbial population that is capable of performing a series of biochemical transformations on the pesticide. Hankin & Hill (1978) studied the proportion of bacteria in agricultural soils that were able to produce degradative enzymes and concluded that the actual numbers of bacteria could not be used as guarantee for a particular soil being able to degrade a particular substrate but the quantitative enzymic activity of the bacteria must be taken into account.

## 1.4 The Study of the Degradation of Xenobiotic Compounds

### 1.4.1 Traditional Methodology

Traditionally, microbioligists have studied the fate of xenobiotic compounds in the environment by initially isolating an organism that was capable of utilising the selected compound followed by a procedure, usually involving solid growth media, to isolate and identify the organism capable of utilising the substrate. The next steps were to culture the organism in sufficient quantities in order to study the biochemical basis of degradation, the regulation of the degradative pathway and a study of any novel features that the metabolism may exhibit.

This methodology has, and will, provide valuable information concerning the growth of organisms and the degradation of xenobiotic compounds. However, almost without exception, this type of study does not reflect the fate of the compound in the natural environment which is a dynamic, open system.

### 1.4.2 Batch Cultures

Batch cultures are considered closed cultures since there are parts of the system which cannot enter and leave freely (Pirt, 1975). A closed system is, thus, always in a transient state since, at any one time, the parameters that define the system, e.g. substrate concentration, biomass, will be different compared with an interval of time, dt, prior to the event. In a closed system the growth rate must tend towards zero.

### 1.4.3 Degradation by Pure Cultures

A wide variety of degradations in pure cultures, both in organism and compound utilised, have been reported. Pollero & Pollero (1978) have examined the degradation of DDT by a soil amoeba. Berg (1978) described the degradation of cellulose and cellulose production by *Phialophora malorum*. Jones & Carrington (1972) studied the growth of a pure culture of an unspecified organism on thiocyanate. Creosote has been used as a substrate for *Pseudomonas sp.* and *Aeromonas sp.* (Line, 1977). An *Alcaligenes sp.* has been isolated which can degrade and grow well on the

styrene dimer both in its cyclic form (1-methyl-3-phenylindane) and the straight chain form 1,3-diphenyl-1-butene) (Tsuchii, Suzuki & Takahara, 1977). Malathion (Bourquin, 1977), triazine (Zeyer, Bodmer & Hutter, 1978), alkylphenol polyethoxylates by a Nocardia sp. (Baggi, Bereta, Galli, Scolastico & Treccani, 1978), para aminobenzene by Bacillus subtilis (Horitsu, Takada, Idaka, Tamoyeda & Ogawa, 1977), benzyl penicillin by Pseudomonas fluorescens (Johnsen, 1977) are a few of the very many examples reported in the literature. Dagley (1971, 1975) has reviewed the catabolism of aromatic compounds by microorganism and the problem of environmental pollution by toxic or recalcitrant compounds.

### 1.4.4 Continuous Culture

Continuous-flow culture offers many advantages over batch culture for observing the degradation of pesticides by microorganisms. These may be summarised as follows.

(1) The physiology of the microorganisms being studied can be controlled directly by controlling the dilution rate of the chemostat since, at a steady state $\mu$, the specific growth rate is equal to D the dilution rate.

(2) Investigations of many generations of microorganisms can be made because the chemostat is an open system. The theory of continuous culture is detailed in section 1.4.5.

(3) Trace quantities of a more recalcitrant compound can be added to populations growing on some carrier substrate and in this way more organisms can be screened for any activity against the recalcitrant compound. This use of a carrier carbon source is termed cometabolism and is further explained in section 1.4.6.

(4) The investigation of mixed populations can be more easily studied and, because of the ability to continue an experiment for may generations, true mixed cultures can be studied, not merely succession of organisms. A true mixed culture may only be obtained if interactions, other than competition for growth-limiting substrate, occur between the component populations. Mixed cultures are fully discussed in section 1.5

(5) Open cultures are more accurate copies of the natural environment. Only in the most extreme of environments are pure cultures found, and because of the close proximity of species it is inevitable that some form of interactions occur. The chemostat is stirred to maintain an homogenous culture and close proximity of component population must occur.

### 1.4.5 The Theory of Continuous Culture

Consider a vessel of fixed volume, V, having a medium feed rate, F. The volume is kept constant by continual removal of medium at the same rate, F. The time taken to change the volume completely is termed the dilution rate, D, and is equivalent to the flow rate divided by the volume.

$$D = F/V \qquad 1.1$$

The medium contains a single growth-limiting substrate at concentration, $S_r$, which is utilised by the organism for growth. As a result of this growth the concentration of substrate is reduced to s and biomass, x, is formed. The specific growth rate of an organism is dependent on the concentration of the growth limiting nutrient in the medium (Monod, 1942). In a closed system this yields:

$$\mu = \mu max \left[ \frac{S}{Ks + S} \right] \qquad 1.2$$

where $\mu$ is the specific growth rate, S is the concentration of growth limiting substrate, Ks is the saturation constant numerically equal to the substrate concentration which gives $\mu = \mu max/2$.

The biomass balance in a chemostat over a period of unit time may be expressed as the amount of biomass produced minus the amount of culture washed out from the vessel, or

$$\frac{dx}{dt} = \mu x - Dx \qquad 1.3$$

that is:

$$\frac{dx}{dt} = x(\mu - D) \qquad 1.4$$

Clearly then, there are three states that can occur:

(1) if $\mu > D$ then dx/dt is positive and x will increase

(2) if $\mu < D$ then dx/dt is negative and x will decrease and washout

(3) if $\mu = D$ then dx/dt is zero and x will remain constant.

This third option with no change in biomass concentration is termed a steady state, and under steady state conditions the physiology of the organism will remain constant.

A similar growth limiting substrate balance equation can be formulated, as 1.2, for continuous culture in a steady state. The change of substrate over a unit time period may be defined as the rate of input of fresh substrate minus the amount used by the growing population minus the amount of substrate washed out, or:

$$\frac{ds}{dt} = DSr - D_s - \frac{\mu x}{Y} \qquad 1.5$$

where Y is the growth yield, defined as the quantity of biomass produced as a result of the utilisation of unit amount of growth limiting substrate.

Thus, in a steady state, we can define the biomass concentration as $\tilde{x}$ and the growth limiting substrate concentration as $\tilde{S}$ where:

$$(\mu - D)\, x = 0 \qquad 1.6$$

and

$$D(S_r - s) - \mu x/Y = 0 \qquad 1.7$$

to obtain values for x and s substitute equation 1.2 in 1.6 gives

$$\left(\mu - \frac{\mu max S}{(s+K_s)}\right) x = 0 \qquad 1.8$$

or $\tilde{S} = \frac{Ks}{(\mu max - \mu)}$ 1.9

by substituting $\mu = D$ this resolves to

$$\tilde{S} = KsD / (\mu max - D) \quad 1.10$$

Similarly, for equation 1.7 it can be shown that

$$\tilde{x} = Y (S_r - S) \quad 1.11$$

or

$$\tilde{x} = Y [Sr - KsD/(\mu max - D)] \quad 1.12$$

There are many reviews of the role of continuous culture in the study of microbial populations. (Jannasch & Mateles, 1974; Parker, 1966; Pirt, 1975; Harder, Kuenen & Matin, 1977; Howell, 1976).

### 1.4.6 Cometabolism

The term co-oxidation was first used by Leadbetter and Foster (1960) and described as "Non-growth hydrocarbons are oxidised when present as co substrates in a medium in which one or more different hydrocarbons are furnished for growth". They concluded that the "inability to grow at the expense of a particular hydrocarbon is not a consequence only of an organisms inability to attack the substrate but may be due to its inability to assimilate the oxidation products". Horvath (1972) has reviewed the role of microbial cometabolism in the degradation of organic compounds in nature. A cometabolic system has been defined where cometabolism of meta-fluorobenzoate (MFB) was carried out in basal salts solution. The MFB did not support growth of the organisms alone but were subject to ring cleavage. The rate of cleavage increased when glucose was added as a co-substrate (Horvath & Flathman, 1976). The theoretical implications of cometabolism have been studied (Hulbert & Krawiec, 1977). Where the carrier substrate is a chemically similar compound cometabolic techniques are termed "analogue enrichment" since the carrier compound is an analogue of the recalcitrant compound (Focht & Alexander, 1970).

## 1.5 Mixed Cultures

Recently it has become apparent that it is possible to isolate mixed cultures of microorganism which will remain highly stable under a variety of environmental perturbations (Slater, 1978; Slater & Somerville, 1979; Slater & Bull, 1978).

It has been stated that "only if interactions, other than competition for the same limiting substrate, occurs between species, may they attain a mixed culture steady state, (Jannasch & Mateles, 1974). These interactions may be of various types and an explanation of these types is given in section 1.5.1 and subsequent sections.

Although the behaviour of mixed cultures has been extensively reviewed (Meers, 1973; Fredrickson, 1977; Veldkamp, 1977), there has been no attempt to establish whether the same associations, that have been isolated under highly selective laboratory conditions, exist in the same way in nature. Also, because of the highly selective conditions, no attempt has been made to study what effect these conditions have on any other organism selected against, which might have a role of importance in the natural environment.

Clearly, these problems will be difficult to resolve but they should be borne in mind during any study of mixed cultures.

### 1.5.1 Associations based on Nutrient Requirement

Some microbial associations have been isolated where one of the component populations cannot synthesise an essential growth factor and, a second member produces this factor as a byproduct of growth in excess of its requirements of this particular product.

Jensen (1957) isolated such a community which comprised of an unidentified bacterium and two Streptomyces sp. The bacterium was unable to produce vitamin B12 and was dependant on the streptomycetes for a supply of B12. The streptomycetes, however, were unable to dechlorinate trichloroacetate, the sole carbon source, but the bacterium was able to.

Other single vitamin requiring communities have been isolated (Nurmikko, 1954; Yeoh, Bungay & Krieg, 1968).

Linton & Buckee (1977) isolated a five membered community growing on methane, with only one methane utilising organism. The four secondary organisms removed inhibitory products, such as methanol and formate, which are normally inhibitory to the primary utiliser, Methylococcus sp.

The removal of toxic products from the growth media by secondary organisms has been demonstrated in many cases, and in some cases the primary utiliser, of the compound being studied, could grow alone on the substrate. Wilkinson, Topiwala & Hamer (1974) described a four membered community consisting of a Pseudomonas sp. which was able to grow on methane in pure culture, and a Hyphomicrobium sp. which removed inhibitory methanol as it was produced by the oxidation of methane. The other two species in the community had indeterminate roles.

Another community isolated, growing on methanol, also required the removal of toxic products by secondary organims (Cremieux, Chevalier, Cambert, Dumenil, Parlour & Ballerini, 1977). This community was proposed as a basis for single cell protein production. The growth parameters of the same mixed culture have also been studied (Ballerini, Parlour, Lapeyronnie & Sri, 1977).

### 1.5.2 Microbial Communities Based on Combined Metabolic Attack

Microbial communities based on combined metabolic attack show examples of interactions which are absolutely necessary for the degradation of the xenobiotic compound studied. Component populations in the community are incapable of transforming the compound totally, whereas collectively the metabolic capability is present. A major consequence of this in the study of biodegradation is that communities may have been unidentified as having activity against a certain compound due to the failure of the researcher to identify a pure culture capable of performing the required transformation. The literature contains examples of enrichment experiments that might have resulted in a microbial community being isolated which performed the desired function, but due to the search for pure cultures this function was overlooked. (Bounds, Magee & Colmer, 1969; Campacci, New & Chan, 1977).

Gunner & Zuckerman (1968) described the synergistic activity between an Arthobacter sp and a Streptomyces sp on the insecticide Diazinon. Separately neither organism could grow on diazinon as sole source of carbon + energy. The nature of the interactions was not determined.

Baggi et al (1978) showed how the sequential action of two microorganisms achieved the total mineralisation of the surfactant alkylphenol ethoxylate. Horwitz et al (1974) isolated a three membered mixed population that was capable of the degradation of crude oil. Strain UP.2 was capable of degrading the paraffin component of the crude oil. Two other strains could grow well in UP.2 deleted oil indicating that compounds necessary for their growth were supplied by UP.2 upon metabolism of the paraffins. UP.2 was the only organism capable of oil emusification, the initial step in biodegradation of the oil. Strain UP.3 and UP.4 only reached their maximum growth yields in the presence of UP.2 indicating a metabolic interaction of some sort between the populations.

Pawlowsky & Howell (1973 a,b,c) have studied the mixed culture bio-oxidation of phenol and the growth parameters of that system but have failed to determine the exact nutritional relationship between the interacting organisms.

### 1.5.3 Microbial Communities based on Cometabolism

The theory of cometabolism has been described in section 1.4.6. It is now clear that cometabolism will be shown to play an important role in the degradation of recalcitrant compounds since in this mode it is more easy for laboratory experiments to mimic the natural environment.

In nature it is most likely to occur when one organism, oxidising a particular compound present in the environment, generates a compound, which it is unable to oxidise further but a second organism can use as a source of energy. In this way an interacting microbial community may be established.

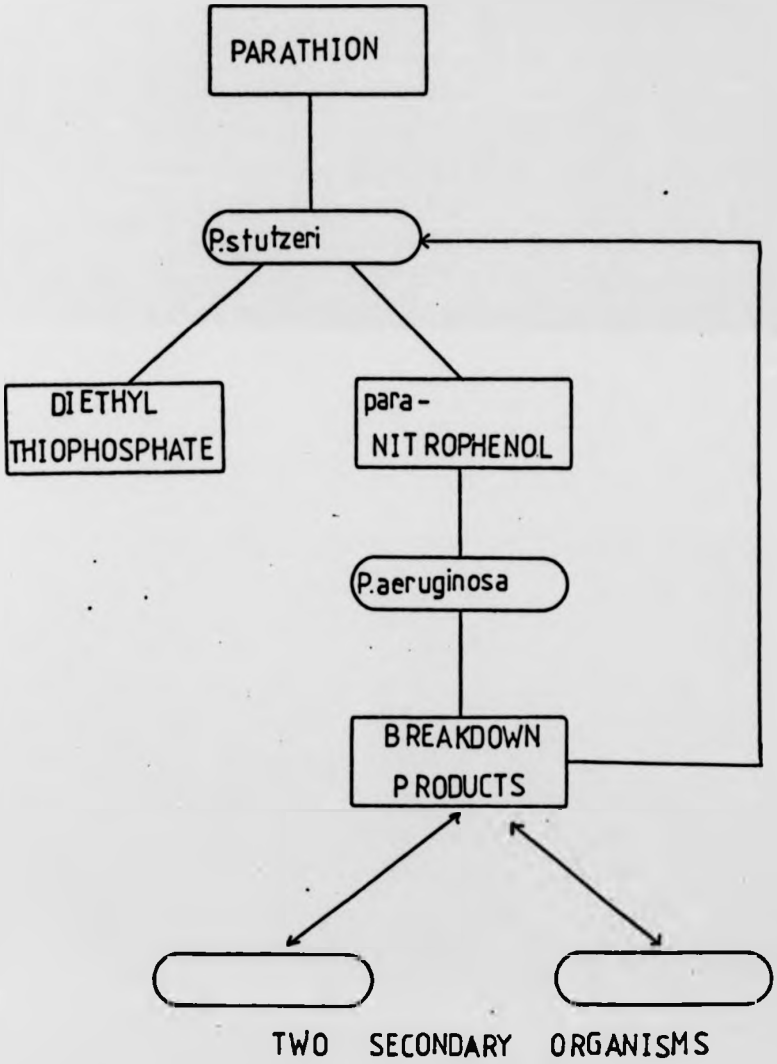
The most detailed examination reported so far concerns the degradation of the organophosphorus insecticide, parathion (0,0-diethyl-0-paranitrophenyl P-phosphothioate) (Munnecke & Hsieh, 1974). The culture was isolated from sewage and soil using glucose as a carrier carbon source. In the initial enrichment cultures there were at least nine different organisms, which may reflect the unspecificity of the carrier substrate. A detailed examination of one of the closely interacting communities by Daughton & Hsieh (1977) revealed a very interesting situation. The community contained four organisms, none of which was capable of growing in pure culture on parathion. One of the organisms, however, Pseudomonas stutzeri did possess an active parathion-hydrolysing enzyme generating diethylthiophosphate and paranitro phenol, neither of which were products which it could oxidise. Another member of the community, however, Pseudomonas aeruginosa was capable of degrading paranitrophenol.

Thus it was essential for the degradation of parathion to continue that some substrate be supplied to the P. stutzeri in order that it might perform a cometabolic attack on the parathion. This relationship is shown in Figure 1.1. Clearly the two secondary organisms must have supplied some compounds to the breakdown products pool since a "climax ecosystem" of this kind will, and did, show a high resistance to alteration of its stability.

Another interesting fact to be observed here is that diethyl thiophosphate was not attacked at all and built up in molar relationship to the parathion added. It is interesting to speculate why no organism is present in the system which has activity against this compound.

The possibility of constructing a community which has the metabolic capabilities desired would be an important step in understanding the relationships which occur in nature. For instance, if an organism were found which could utilise diethyl thiophosphate and it was added to the "parathion community" would a second community evolve which was capable of total mineralisation of the parathion?

Figure 1.1 Symbiotic relationship in the degradation of parathion.


PARATHION
P.stutzeri
DIETHYL THIOPHOSPHATE
para-NITROPHENOL
P.aeruginosa
BREAKDOWN PRODUCTS
TWO SECONDARY ORGANISMS

### 1.5.4 Communities with more than one Primary Utiliser

A feature more recently found in some communities isolated is the presence of more than one primary utiliser. An organism is said to be a primary utiliser if it can grow on the substrate in pure culture.

Conventional chemostat kinetics predict that in such a case the organism that has a faster maximum growth rate or a higher substrate specifity will outgrow the other population. This means that there must be interactions between the primary utilising populations in order to stabilise the community and allow a steady state to develop.

The first community to exhibit this phenomenon was that described by Senior, Bull & Slater (1976). The community mineralised the herbicide Dalapon (2,2-dichloropropionic acid) and consisted, initially, of three primary utilisers and four secondary utilisers. One of the secondary utilisers, a pink budding yeast, was soon lost from the community as the growth rate of the community was increased. The other six members remained tightly bound together for a very long period of time despite environmental changes imposed upon it, including change in growth rate, pH, temperature and influent substrate concentration.

An interesting feature of this community was that, after several thousands of hours growth in a chemostat, one of the secondary utilisers, Pseudomonas putida, acquired the ability to degrade the Dalapon, that is, the community developed a fourth primary utiliser.

The generation of this new Dalapon utiliser is important in that had the community not existed, the particular strain of P.putida could not have been exposed to the Dalapon for such a length of time and would, thus, not have had the opportunity of evolving a metabolic activity against the Dalapon. Another interesting problem is that supposing the original community was a "climax ecosystem" (a stable, usually multimembered, community resulting from succession under stable climatic and environmental conditions (Kelly, 1978)) then it should show a high resistance to alteration of its stability by an intruder, the ease with

which "the intruder" formed a second "climax ecosystem" suggests that these communities may be more common in nature than first thought.

A mixed culture growing on formate as the limiting substrate, with two primary utilisers has also been described (Sinerez & Pirt, 1977). The mechanism of the interaction of the two primary utilisers was not determined.

### 1.5.5 Miscellaneous Communities

An example of how tight a microbial community may be is highlighted by the organism Chloropseudomonas ethylica (Gray, Fowler, Nugent, Rigopoulous & Fuller, 1973). This organism was found to be symbiotic association of two species, a nonmobile green photosynthetic bacterium and a colourless mobile bacterium. All attempts by Gray et al (1973) to isolate mobile photosynthetic bacteria failed, but, prior to 1973, the mixed culture was considered to be a single species.

Bryant, Wolin, Wolin & Wolfe (1967) have isolated another "single organism" that in fact turned out to be a mixed culture. Methanobacillus omelianskii was found to consist of an S organism and a Methanobacterium sp. In this case the S organism utilised ethanol under anaerobic conditions and generated acetate and hydrogen ions. The organism required a sink to dispose of these reducing equivalents, and it was provided by the methanogen utilising $CO_2$ as its electron acceptor and generating methane. An important feature of this community is that the potential source of the hydrogen ions is utilised for maximum biomass production.

A novel community was isolated by Osman, Bull & Slater (1976) in which the growth parameters of the individual organisms were altered by the community structure. The community was grown on orcinol (3,5-dihydroxytoluene) and comprised a primary utiliser and two secondary organisms. It was thought that the secondary organisms grew on acetate as this was excreted by the primary utiliser during exponential growth on orcinol. The substrate affinity, Ks (see section 1.4.5), of the community was lower for the complete community than for the primary utiliser when growing on orcinol alone. Thus the mixed culture had achieved a more

competitive state, than the pure culture of the primary utiliser, resulting in a stable mixed culture.

## 1.6 The Herbicide Lontrel

Lontrel is the Dow Chemical Co. trademark for 3,6-dichloropicolinic acid. It has been introduced for the control of phenoxy-tolerant brush, woody rangeland and deep rooted perennial broadleaf species. Crops tolerant to Lontrel and its formulations are cereals, maize, flax, grasses and brassicae such as oil seed rape. It is a growth regulator herbicide and is readily absorbed by the roots and leaves and translocated throughout the plant. In susceptible plants it induces auxin like responses resulting in etiolation and subsequent death.

Clearly this is an important herbicide in terms of increased food production due to its selective nature of inhibiting competitors to cereal crops. In order to maintain the quality of the environment it is essential that herbicides of this type are mineralised and not merely degraded.

The Dow Chemical Co. have performed initial degradation studies on 36DCPA and concluded that the herbicide was degraded in the soil but were unable to elucidate any degradation pathway (Dow Chemical Co., 1976). They suggested that degradation might occur via a 3-chloro-5,6-dihydroxypicolinate compound. Pik, Peake, Strosher and Hodgson suggested that the 6-hydroxy derivative might be a potential breakdown product but since they could find little or no accumulation of this compound in the soil they concluded that ring cleavage must be the rate limiting step in the degradation sequence.

Houghton and Cain (1972) have studied the microbial metabolism of the pyridine ring and concluded that for all the pyridines studied pyridinediols were formed. In the case of 2-hydroxy and 3-hydroxypyridines the diol produced was pyridine-2,5-diol, i.e. hydroxylation diagonally opposite the initial hydroxylation.

Dagley and Johnson (1963) have stated that oxidation of picolinate occurs by hydroxylation at the 6 position.

It might be concluded, therefore, on available evidence that the degradation of 36DCPA may occur by hydroxylation at the 6 position, with release of chloride ions, followed by hydroxylation at the 3 position, with release of chloride ions. The dihydroxypicolinate may then be degraded in a manner similar to gentisate as described by Dagley (1971).

## 1.7 Aims of this Study

The aim of this study was to determine the fate of 3,6-dichloropicolinic acid in the soil. In order to do this it was necessary to:

(1) Study the activity of microorganisms in 36DCPA treated soil.

(2) Try to establish an organism or microbial community that was capable of degrading 36DCPA and utilising the herbicide as a source of carbon and energy.

(3) Examine the possibility of a cometabolic degradation.

and upon isolation of a degrading population to try to establish the mechanism by which 36DCPA was mineralised and to try to extrapolate this laboratory situation to the natural environment.

CHAPTER 2

# MATERIALS AND METHODS

## 2.1 GROWTH MEDIA

In all experiments organisms were grown in a defined medium comprising three components, namely basal salts, trace elements and a carbon source.

### 2.1.1 Basal Medium Composition

The basal mineral salts contained, in glass distilled water, ($gl^{-1}$): $K_2HPO_4$, 1.5; $NaH_2PO_4$, 0.5; $(NH_4)_2SO_4$, 0.5; $MgSO_4.7H_2O$, 0.2.

The trace elements solution contained, in glass distilled water, ($gl^{-1}$): EDTA, 12; $Na_2SO_4$, 10; NaOH, 2; $FeSO_4.7H_2O$, 2; $MgSO_4.7H_2O$, 1; $ZnSO_4.7H_2O$, 0.4; $MnSO_4.7H_2O$, 0.4; $CuSO_4.5H_2O$, 0.1; $NaMoO_4.2H_2O$, 0.1; and 0.5ml concentrated $H_2SO_4$.

Both these solutions were autoclaved separately at 15lbs $in^{-2}$ for 15 minutes. The defined medium contained 10ml of trace elements solution per litre of basal salts, added asceptically after sterilisation.

Carbon sources were prepared as 10% (w/v) solutions and neutralised by the addition of solid NaOH. Halogenated carbon compounds were filter sterilised to prevent thermal dechlorination whilst non-chlorinated compounds were autoclaved at 15lbs $in^{-2}$ for 15 minutes. Carbon sources were normally added aseptically to the growth medium to give a final concentration of 0.5g carbon $l^{-1}$.

### 2.1.2 Other Media

Defined media were solidified by the addition of 1.5% (w/v) agar.

Nutrient agar (Lab M, Salford, Lancs, UK) was made up according to the manufacturers recommendation of $31gl^{-1}$ in glass distilled water.

When 3,6-dichloropicolinate (36DCPA) was used as a nitrogen source the basal mineral salts were made up without $(NH_4)_2SO_4$ and 36DCPA was added to a final concentration of 0.11g nitrogen $l^{-1}$.

## 2.2 ORGANISMS

The organsims used in this study were all new isolates from soil enrichments and were identified with the collaboration of the Torry Research Station, (National Collection of Industrial Bacteria), Aberdeen. The identification of the isolates is detailed in section 5.1.2.

### 2.2.1 Maintenance

Organisms were maintained on slopes of defined medium solidified with 2% (w/v) agar at 4°C. Organisms were also stored at -18°C in 50% (v/v) sterile glycerol. For the latter method of storage, exponentially growing organisms were harvested by centrifugation at 23,000g for 10 minutes, washed in sterile ice-cold 0.2M phosphate buffer (Gomori, 1955), recentrifuged, and finally resuspended in the same buffer. The suspension was mixed with an equal volume of sterile glycerol, dispensed into sterile bijou bottles and stored at -18°C.

## 2.3 GROWTH OF ORGANISMS

### 2.3.1 Closed Culture Systems

250ml conical flasks containing 100ml defined medium were incubated at 30°C on an LH Engineering Mk V rotary shaker (Stoke Poges, Bucks, UK). Where direct measurements of absorbance were required 250ml sidearm conical flasks were used. Growth was monitored by measuring the absorbance in a Corning colorimeter fitted with an orange filter (peak transmission 600nm).

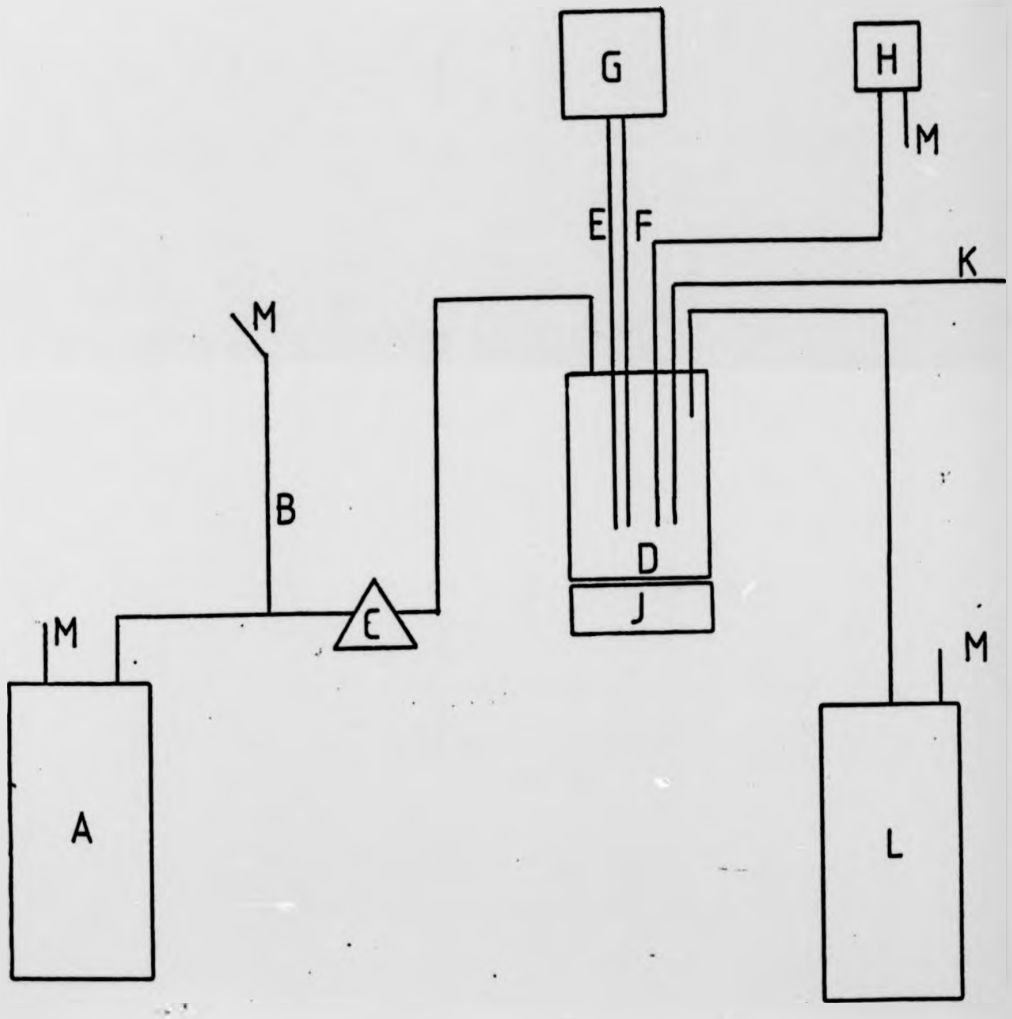
## 2.3.2 Open Culture Systems

1 litre growth vessels were used in all continuous-flow culture experiments. Two types of fermenter system were used, namely, an LH Engineering Series 500 fermenter units (LH Engineering, Stoke Poges, Bucks, UK) and a system based on Quickfit glass equipment (Figure 2.1). The working volumes of the vessels were 0.85l and 0.8l respectively.

The only significant difference between the two fermenters was the culture vessel lid. In the Quickfit system the lid carried five inlet/outlet ports, was made of glass and was secured to the vessel by a metal spring ring. In the LHE Series 500 fermenter the lid was stainless steel and carried additional ports for variations on the simple chemostat functions, such as ports for pH control, and antifoam control. The fermenter units were autoclaved at 15lbs $in^{-2}$ for 40 minutes. The fermenter vessel contained 100ml of minimal medium during autoclaving to ensure total sterilisation of the vessel. 100ml mid-exponential phase closed cultures were used to inoculate the fermenter. Waste culture was removed by maintaining a slightly raised pressure in the vessel thus forcing out culture and keeping the volume in the culture vessel constant. The temperature of the culture was maintained at 30°C by either the temperature control module (LHE Series 500 type) or the cool/warm finger connected to a Churchill water heater. A homogenous culture was maintained by stirring at 750 rpm (LHE Series 500) or 600 rpm (Quickfit system) with a magnetic stirrer. Air was sparged through the culture at one volume per volume of culture per minute. Samples were taken from the vessel by closing the overflow pipe, resulting in an increase of air pressure within the vessel, and so forcing culture through the sample port when opened. Medium flow rate was determined by monitoring the time taken for a known volume of culture to be pumped into the culture vessel and calculated in terms of ml $h^{-1}$. A steady state was said to be achieved after a minimum of three culture volumes had passed through the culture vessel and the culture absorbance had remained constant for that period of time.

Figure 2.1 Schematic representation of the LHE series 500 and the Quickfit continuous-flow culture apparatus.

A, Fresh medium vessel; B, Graduated tube for measuring flow rate; C, Watson Marlow flow inducer D, Fermenter vessel; E, Heating element; F, Thermocouple; G, Temperature control module; H, sample port; J, Stirrer; K, Air inflow; L, Waste pot; M, Air pressure equalisation filters. In the Quickfit system E, F, and G were substituted for a cold/warm finger and a Churchill water circulator to maintain the temperature.


G
H
M
E
F
K
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B
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J
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M
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A
L

## 2.4 ENRICHMENT SYSTEMS

Several methods of enrichment were used to isolate a microorganism or a group of microorganisms that were capable of growing on 3,6-dichloropicolinate (36DCPA) or other carbon sources.

### 2.4.1 Direct Enrichment

For direct enrichment 5g of a peaty loam soil that had been sprayed once with 36DCPA at field application rates (1.2kg $ha^{-1}$, supplied by The Dow Chemical Co., Kings Lynn, Norfolk, UK) was placed in a sterile flask containing defined medium with 3,6-dichloropicolinate at a concentration of 0.5g carbon $l^{-1}$ as a sole source of carbon and energy. The flask was shaken at 30°C and sampled regularly for organisms which could grow on solid medium containing 36DCPA at 0.1, 0.25 and 0.5g carbon $l^{-1}$.

### 2.4.2 Soil Columns

An adaptation of the method used by Macura & Málek (1958) was developed to continually percolate soil with 36DCPA defined medium.

#### 2.4.2.1 Preparation of Soil Particles

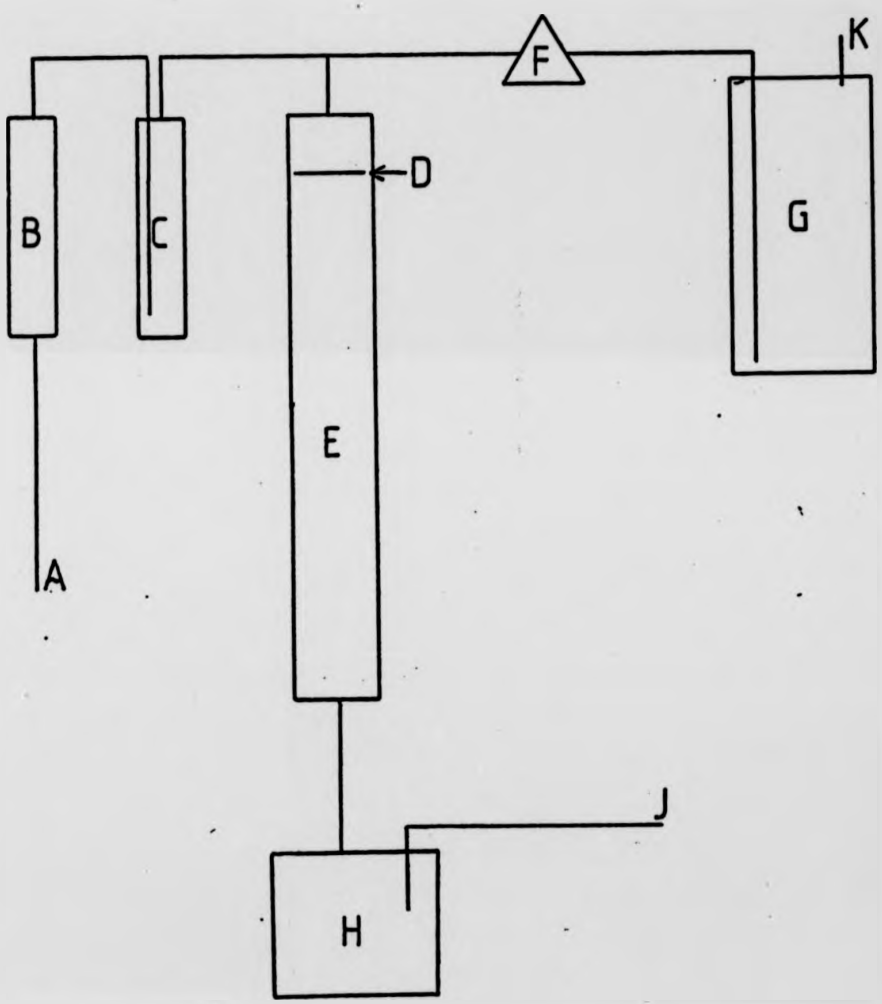
Soils were air dried for two days at room temperature and particulate matter betweem 3mm and 5mm diameter was picked out to fill the columns.

#### 2.4.2.2 Continuous-Flow Enrichment Apparatus

Figure 2.2 shows a schematic representation of the apparatus. The influent reservoir was a 10l bottle containing defined medium supplemented with organic carbon sources. Medium was pumped onto the column by a Watson Marlow flow inducer (MHRE 2) (Falmouth, Cornwall, U.K.) at a constant rate of 8ml $h^{-1}$. Humidified carbon dioxide-free air was fed to the column at 50ml $min^{-1}$. The system was maintained at room temperature.

Figure 2.2 Schematic representation of the soil enrichment columns.

A, Air in flow; B, Manometer; C, Humidifier; D, Nylon Gauze,; E, Column packed with soil particles 3-5mm diameter; F, Watson Marlow flow inducer; G, Medium reservoir; H, Effluent reservoir; J, Waste; K, Air pressure equalisation filter.


F
K
B
C
D
G
E
A
J
H

2.4.2.3 Sampling Procedure

Samples for microbial population analysis were taken from the effluent culture or directly from the soil column. 0.1ml of culture was plated directly onto agar plates containing defined medium with a variety of carbon sources. In addition 1ml samples were inoculated into shake flasks containing defined media with a variety of carbon sources, incubated for 1 week at 30°C and 0.1ml of this culture was plated out.

Soil was sampled from the column by removing soil aggregates with a sterile inoculating loop. The aggregates were shaken in sterile 0.2M phosphate buffer (section 2.2.1) and the suspension was treated in the same way as the column effluent.

2.4.3 Analogue Enrichment. (Focht & Alexander, 1970)

Samples of soil from the soil column (section 2.4.2) and samples of untreated soil were used as an inoculum into a 1l fermenter (LHE series 500 system) containing defined medium with unsubstituted picolinate as the sole source of carbon at 0.5g carbon $l^{-1}$. The samples were grown as a closed culture for several days before initiating a flow of fresh medium. To expose organisms capable of growing on picolinate to 36DCPA a method was devised having two media reservoirs connected by a Y piece. This enabled the medium flowing into the culture vessel to be changed from picolinate alone to a mixture of picolinate plus 36DCPA by loosening and clamping off the respective tubing.

## 2.5 MINERALISATION OF [$^{14}$C]-3,6-DICHLOROPICOLINATE

Mineralisation of [$^{14}$C]-3,6-dichloropicolinate was measured using a method similar to Goswami & Koch (1976). The [$^{14}$C] 36DCPA (with aspecific activity, 10.6 $\mu$Ci $\mu$mol$^{-1}$) was labelled in the carbon 2 and 6 positions and was kindly supplied by The Dow Chemical Co., Kings Lynn, Norfolk, U.K.

### 2.5.1 Degradation Apparatus

Figure 2.3 shows a schematic representation of the apparatus used to measure the degradation of [$^{14}$C]-36DCPA. Air was sparged through water to humidify it and through potassium hydroxide to remove any carbon dioxide present. The air was sterilised by passing through a sterile glass fibre filter. [$^{14}$C]-carbon dioxide formed by degradation of the herbicide was forced out of the reaction vessel, through a drying bottle and into 8ml of a carbon dioxide trapping solution (section 2.5.3) by the incoming air. The apparatus was maintained at 30$^{o}$C. The reaction vessel was not continually flushed with air because this caused an unacceptably high amount of absorption reagent into the vapour state, and hence was lost. The vessel was flushed for at least 24h with air before a sample was to be measured for $^{14}CO_2$.

### 2.5.2 Preparation of Soil

Soils were collected, sealed in polythene bags to maintain the collection moisture content and stored in cardboard fibre boxes at 4$^{o}$C. 100g of soil was weighed into a 250ml conical flask and a field application of 2,6-[$^{14}$C]-36DCPA, namely 25 µg, that is, 0.25 µg g$^{-1}$ (0.25 p.p.m.) was added. An identical flask was established with soil that had been heat sterilised at 160$^{o}$C for 16h, and reconstituted to the correct moisture content by the addition of sterile distilled water. Table 2.1 shows the relevant data on soils used as supplied by the Dow Chemical Co. Ltd.

### 2.5.3 Absorption Reagent amd Scintillant

The carbon dioxide absorption reagent consisted of 30% (v/v) ethanolamine in 2-methoxyethanol. 8ml of the reagent was added to a scintillation vial and was used to trap the carbon dioxide released.

The scintillant used contained 0.02% (w/v) POPOP (1,4di-2-(5 phenyloxazolyl)-benzene), 0.4% (w/v) PPO (2,5 diphenyl-oxazole), 3 volumes of sulphur-free toluene and 2 volumes of ethoxyethanol. 12ml of the reagent was added to each scintillation vial.

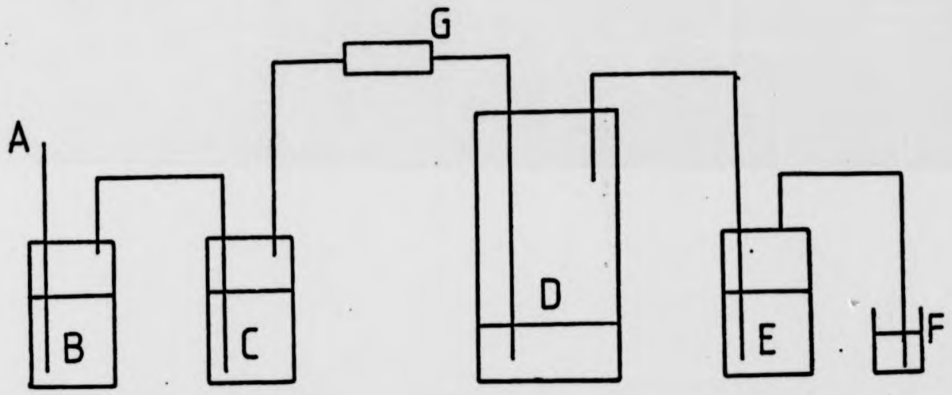
Table 2.1

Data on soil supplied by Dow Chemical Co. for degradation studies.

| Soil | Type | Location | pH |
|---|---|---|---|
| M 46 | Peaty Loam | Mississippi | 6.2 |
| M 47 | Sandy | California | 6.5 |
| M 51 | Peaty | Illinois | 5.8 |
| M 53 | Clay | Montana | 7.7 |

Figure 2.3 Schematic representation of the apparatus used to measure the breakdown of [$^{14}$C]-3,6-dichloropicolinate.

A, Air inflow; B, Humidifier; C, KOH solution; D, Vessel containing soil; E, Concentrated $H_2SO_4$ for drying gas; F, Carbon dioxide absorbing reagent; G, Sterile air filter.


G
A
B
C
D
E
F

### 2.5.4 Measurement of Radioactivity

Samples were counted in a Packard 2425 liquid scintillation counter. The efficiency of counting was measured using the automatic standard incorporated into the instrument.

## 2.6 ANALYSES

### 2.6.1 Estimation of Culture Absorbance

Culture absorbance was measured in a Unicam SP1700 spectrophotometer at 600nm using 3.0ml glass cuvettes with a 1cm light path. (See section 2.3.1 for closed culture determination).

### 2.6.2 Estimation of Culture Biomass

Culture biomass was estimated either by centrifugation or filtering methods.

#### 2.6.2.1 Centrifugation

Duplicate 5ml samples were centrifuged at 3,300g for 10min in preweighed micro-angle centrifuge tubes. The pellet was washed in 3ml sterile distilled water, recentrifuged and dried to a constant weight at 105°C.

#### 2.6.2.2 Filtration

10ml of culture was filtered through predried, preweighed glass fibre (Whatman GF/A) and Millipore (Type HAWP, 0.45μM pore size) filters in series, washed with 3ml distilled water and dried to a constant weight at 105°C.

The biomass of the culture was expressed as mg dry weight $ml^{-1}$. No significant difference was found betweem the two methods.

### 2.6.3 Measurement of Free Chloride Ions in Solution

Chloride ions were determined in a Marius Chlor-o-Counter (F.T. Scientific Instruments Ltd., Tewkesbury, Glouc s., U.K.). Samples (1ml or 5ml) were added to 25ml of a base counting solution (containing 100ml glacial acetic acid and 8.0ml concentrated nitric acid in 1l of glass distilled water) and 1ml gelatin-thymol blue indicator solution (containing 600mg white powder gelatin, 10mg thymol and 10mg thymol blue in 100ml of glass distilled water). Free chloride ions were titrated against silver ions generated coulometrically and resulting in the precipitation of insoluble silver chloride. The titration end point was detected amperometrically by silver electrodes which measured the appearance of free silver ions and the titration time was directly proportioned to the chloride ion concentration. Up to 20ml of sample could be assayed for each 25.0ml of base solution and the detection limit of the instrument was 0.2 µmol chloride ions per sample.

### 2.6.4 3,6-Dichloropicolinate Measurement

36DCPA was analysed by a gas chromatographic method developed by M. Jones, The Dow Chemical Co., Kings Lynn. (Dow Chemical Co., 1975).

#### 2.6.4.1 Preparation of Samples

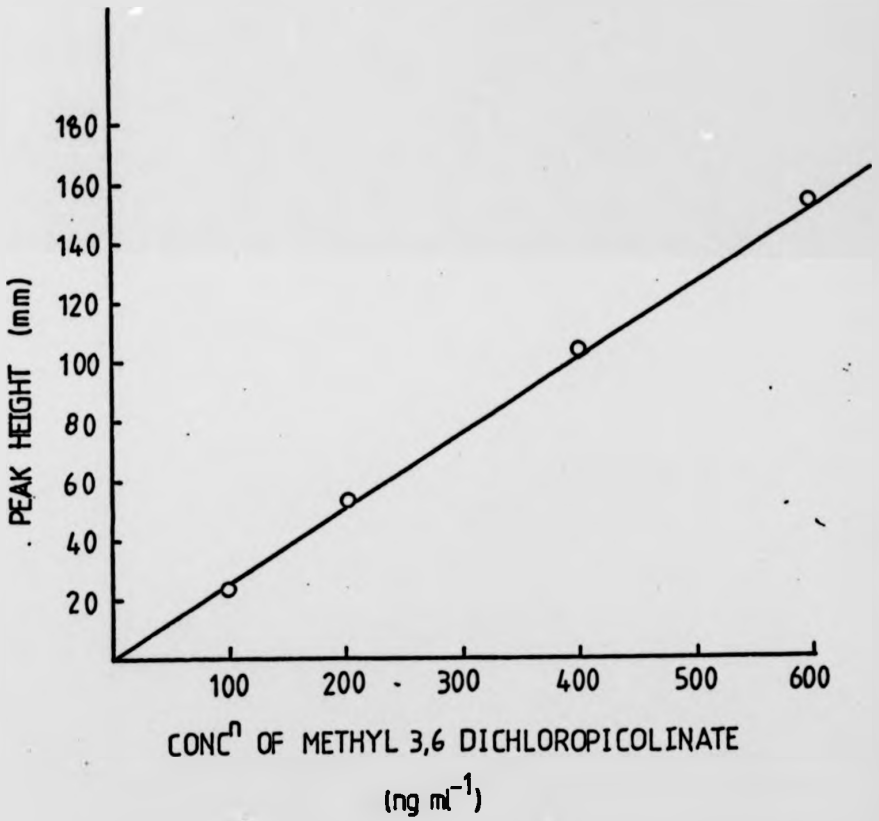
Samples were diluted to within the estimated linear region of the analysis range (5-600 ng $ml^{-1}$) and placed in 20ml of glass distilled water in a 100ml all glass separating funnel. The sample was acidified by the addition of 8ml of 2M sulphuric acid. 5-8g of solid sodium chloride was added to prevent solublisation of the organic phase in the aqueous phase. 3,6 dichloropicolinic acid was extracted with 3 x 10ml aliquots of diethyl ether (Distol grade, Fisons Ltd., U.K.). The ethereal extract was dried with anhydrous sodium sulphate and decanted into a second container. The sodium sulphate was washed with 2 x 2ml aliquots of diethyl ether and the washings transferred to the ethereal solution. This was evaporated to approximately 5ml and an equal

volume of diazomethane reagent (prepared as described in section 2.7.1) was added to methylate the acid. The sample was capped and left for 15min at room temperature to allow complete methylation to occur. The sample was evaporated to dryness using air at room temperature and because of the volatile nature of methyl 3,6-dichloropicolinate, it was essential that there was no elevated temperatures used during this stage of the extraction procedure. The residue was taken up in 30% (v/v) diethyl ether in hexane (both Distol grade) and made up to 10ml in a volumetric flask and analysed by gas chromatography. Standard 3,6-dichloropicolinic acid sample at 100 ng $ml^{-1}$ and 400 ng $ml^{-1}$ were treated to a parallel extraction procedure in order to determine the percentage recovery of 3,6-dichloropicolinic acid for each analysis.

2.6.4.2 Gas Chromatography

The instrument used for gas chromatography was a Perkin Elmer Sigma 3 gas chromatogram fitted with a $^{63}$nickel electron capture detector. The detector was specially designed for the determination of halogenated molecules. The injector and detector blocks were maintained at 305°C since these were the optimum working temperatures for both blocks. The chromatography column used was a 2M 1/4in OD (outside diameter) glass column packed with 10% O.V.225 on Chromasorb Q (Perkin Elmer, Beaconsfield, Bucks., U.K.). A glass column was used because it was found that the pyridine nucleus of the molecule was attracted to, and attached to, a metal column (M. Jones, Personal communication). The column oven was maintained at 195°C. The optimum flow rate of carrier gas (oxygen-free nitrogen) was 60ml $min^{-1}$. 1.0 or 0.5 µl samples were injected into the column and a standard curve was plotted for each extraction. Figure 2.4 shows a typical extraction curve. An average of two peak heights was taken to allow for errors in the injection process.

Figure 2.4 Typical standard curve of gas chromotographic analysis of methyl 3,6-dichloropicolinate.


180
160
140
120
100
80
60
40
20
PEAK HEIGHT (mm)
100
200
300
400
500
600
CONCⁿ OF METHYL 3,6 DICHLOROPICOLINATE
(ng ml⁻¹)

## 2.7 ORGANIC SYNTHESES

### 2.7.1 Preparation of Diazomethane

Ethereal alcoholic solutions of diazomethane were prepared in the following manner. The compound was made behind an explosive screen and in an efficient fume cupboard due to the explosive and toxic nature of the gas.

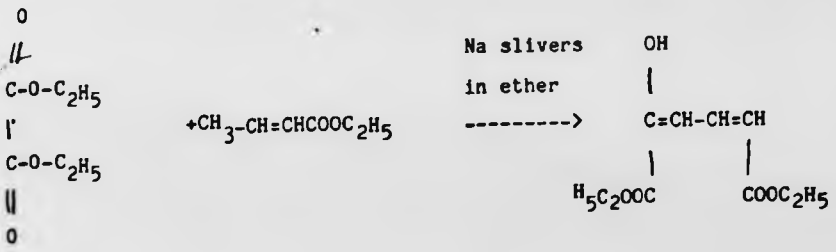
25ml of absolute ethanol were placed in a 100ml reaction flask with 5g potassium hydroxide dissolved in 8ml water. The reaction flask was fitted with a dropping funnel and a downward water cooled condenser. The condenser was attached to two receiving flasks in series, the second of which contained 30ml diethyl ether, and both were kept at $0^{o}C$ using an ice and solid sodium chloride mixture. 21.5g (0.1 mole) of Diazald reagent (N-methyl-N-nitroso-p-toluene-sulphonamide, Aldrich Chemical Co., Gillingham, Dorset, U.K.) was dissolved in 200ml diethyl ether and allowed to drip into the reaction vessel. The vessel was heated to approximately $65^{o}C$ by an electric water bath. An ethereal alcoholic solution of diazomethane was evolved and condensed into the first receiving vessel. The function of the second receiving flask was to absorb any gaseous diazomethane that was not condensed in the first receiving vessel.

When all the Diazald in ether had passed through the reaction flask a further 40ml of diethyl ether was added and the distillation continued until the distillate contained no traces of yellow diazomethane. The ethereal fractions were combined and stored at $-20^{o}C$ in a polystop conical flask. The apparatus used had cleaseal glass joints (Aldrich Chemical Co., Gillingham. Dorset, U.K.) because ordinary ground glass joints could act as an explosion nucleus for the diazomethane.

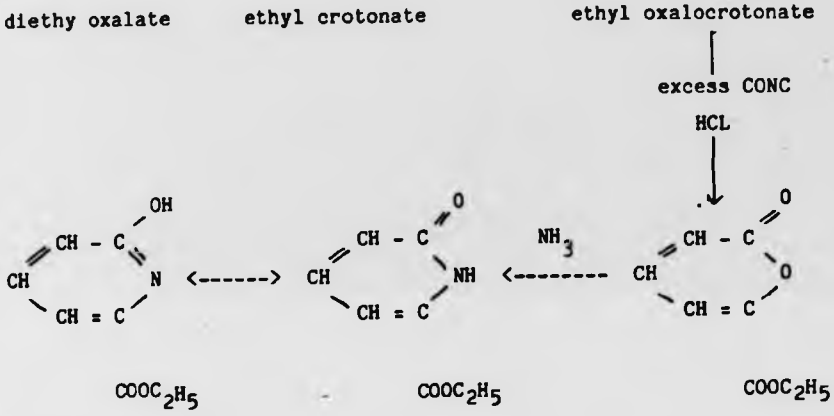
### 2.7.2 Preparation of 6-Hydroxypicolinate

6-hydroxypicolinate was prepared from α-pyrone-6-carboxylic acid (Dagley and Johnson 1963, Lapworth 1901). Figure 2.5 shows the synthesis of 6-hydroxypicolinate.

Figure 2.5 Preparation of 6-hydroxypicolinate

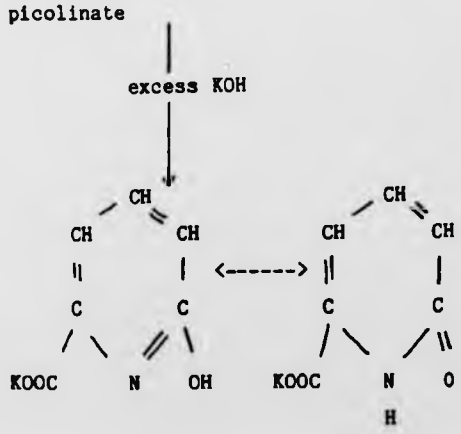



diethy oxalate ethyl crotonate ethyl oxalocrotonate




ethyl-6-hydroxy-picolinate

ethyl coumalin 6-carboxylate




potassium 6-hydroxypicolinate

1 mole of diethyl oxalate and 1 mole of ethyl crotonate were placed in a reaction vessel and covered in diethyl ether. Slivers of sodium metal (3 moles) were added, a reflux water condenser fitted and the reaction vessel placed in an ice bath. The reaction mixture boiled vigorously. The sodium metal removed ethyl groups from the acids and caused condensation of the molecules. When all the sodium had dissolved the reaction vessel was removed from the ice and left for 1 week for crystals of ethyl oxalocrotonate to form. The crystals were filtered under vacuum. A further quantity of ethyl oxalocrotonate was extracted from the filtrate to obtain a high yield (Lapworth 1901) but this was not performed entirely. The crystals were placed in a 500ml reaction vessel and refluxed in the presence of excess concentrated hydrochloric acid for 4h until all the crystals had dissolved. The solution was decolourised by boiling for 2h with decolourising charcoal which was removed by filtration. This step caused the formation of ethyl coumalin-6-carboxylate (ethyl 2-pyrone-6-carboxylate). The solution was evaporated to dryness on a boiling water bath. The solid was taken up in ethyl alcohol and refluxed with a saturated alcoholic ammonia solution (dry ammonia gas bubbled into absolute alcohol at $0^{o}C$ until ammonia evolution indicated that no more could dissolve), adding more ammonia solution when ammonia evolution ceased. This was determined using wet universal indicator paper (B.D.H. Poole, Dorset, U.K.) turning blue in the presence of ammonia. The resultant solution was refluxed for 2h with excess alcoholic potassium hydroxide solution to replace the ethyl group at the ester linkage with a potassium ion. The solution was evaporated to dryness and the water soluble fraction retained. 6-hydroxypicolinate was recrystalised twice from the minimum amount of boiling water as the potassium salt. The purity was checked by determining the melting point of the acid, after acid hydrolysis ($273^{o}C$, Dagley and Johnson, 1963) and proton nuclear magnetic resonance on a Perkin Elmer R12 N.M.R. Spectrometer.

## 2.8 ENZYME ANALYSIS

### 2.8.1 Preparation of Cell Free Extracts

Steady state chemostat cultures (section 2.3.2) were harvested by centrifugation at 13,500g for 10min at 2°C. The pellets were washed once with ice cold 0.2M phosphate buffer pH 7·0 and resuspended in 0.02M phosphate buffer pH 7·0. The suspension was passed twice through a French Pressure cell (Aminco, Maryland, U.S.A.) at $8.3 \times 10^7$ Pa (12.000 lbs in $^{-2}$) at 4°C. The disrupted cell suspension was centrifuged at 42,500g for 30min to remove cell debris and unbroken organisms and provide cell-free extract for enzyme assays. A portion of the extract was stored immediately after centrifugation at -18°C for subsequent protein determination. The extract was stored on ice whilst being used to prevent loss of enzyme activity.

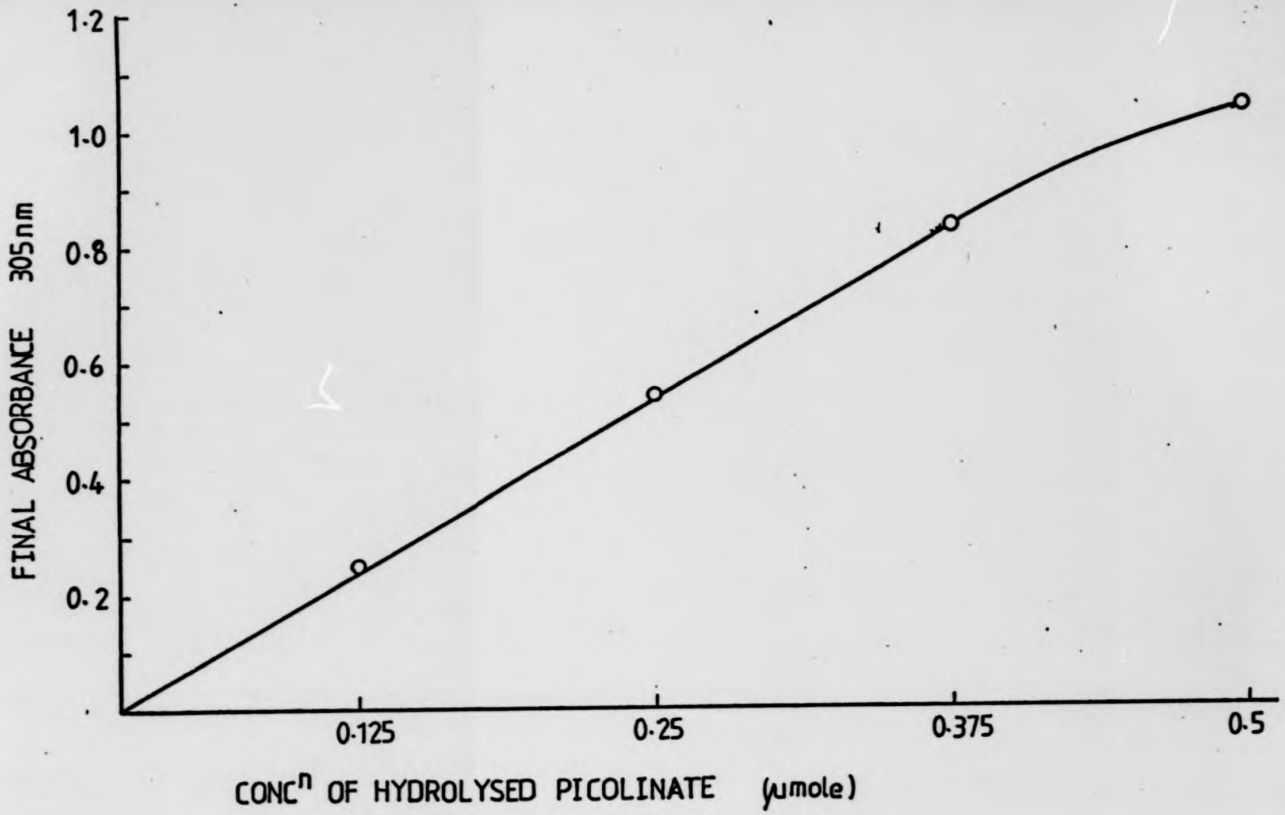
### 2.8.2 Enzyme Assays

All activities were determined in cell-free extract. All assays were carried out at 30°C. Arsenite was used as an inhibitor and minimum concentration of arsenite was calculated in the following manner and was used in all subsequent assay. The activity of an extract was measured under identical conditions excepting the arsenite concentration which was varied from 0.02 µmol $ml^{-1}$ to 0.5 µmol $ml^{-1}$, (final concentration in assay mixture). The minimum inhibitor concentration that was capable of completely halting the reaction was found to be 0.06 µmol $ml^{-1}$ final concentration. A Unicam S.P.1700 recording spectrophotometer was used.

#### 2.8.2.1 Picolinate 6-Hydroxylase

The enzyme was assayed as described by Dagley and Johnson (1963). 1ml of cell-free extract, 2ml of 0.2M tris-maleate buffer pH 8·5 and 0.1ml of 2mM sodium arsenite were equilabrated at 30°C in a 4ml, 1cm path length, quartz cuvette. 0.25ml of picolinate was added to initiate the reaction, and the increase in absorbance at 305nm was monitored. The reference cuvette contained extract, buffer and sodium arsenite but no picolinate. Picolinate 6-hydroxylase activity was expressed as µmole substrate hydrolysed (mg protein)$^{-1}h^{-1}$. The relationship between the change in absorbance and the µmole substrate hydrolysed is shown in Figure 2.6. Low concentrations of picolinate were allowed to be completely hydrolysed and the concomitant rise in absorbance at 305nm was noted (after Dagley + Johnsen, 1963).

Figure 2.6 The relationship between the change in absorbance and the enzyme activity (expressed as μmol picolinate hydrolysed).


1·2
1·0
0·8
0·6
0·4
0·2
FINAL ABSORBANCE 305nm
0·125
0·25
0·375
0·5
CONCⁿ OF HYDROLYSED PICOLINATE (μmole)



in as

### 2.8.3 Measurement of Protein Concentration

Protein was estimated by both the Biuret reaction (Gornall, Baradwill and David, 1949) and by the Folin and Ciocalteu reaction (Lowry, Rosebrough, Farr and Randall, 1951) using bovine serum albumin as a standard protein. The Biuret reaction was used for high concentrations of protein (linear up to 4 mg $ml^{-1}$) and the Folin method for low protein concentrations (50-400 ug $ml^{-1}$).

## 2.9 MEASUREMENT OF OXYGEN UPTAKE

Oxygen uptake was measured using an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) Figure 2.7 shows a schematic representation of the oxygen electrode.

### 2.9.1 Assembly and Calibration of the Oxygen Electrode

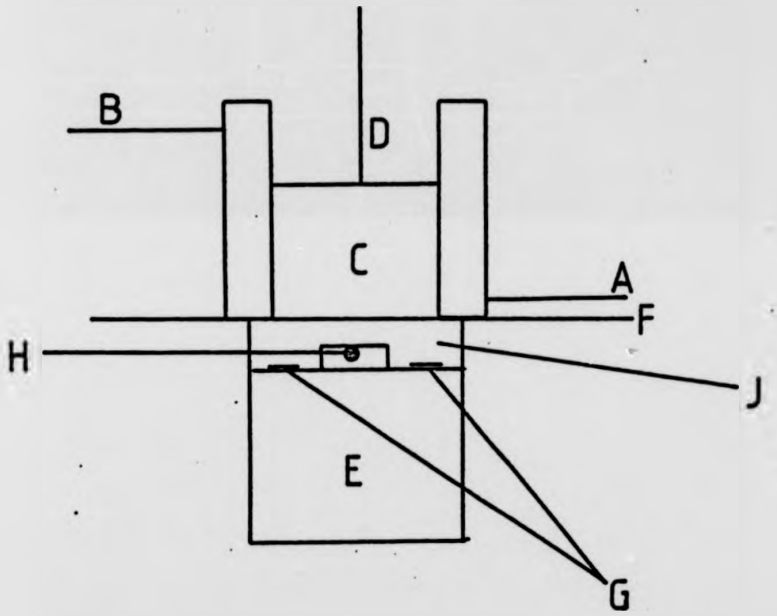
Approximately 4ml of saturated potassium chloride was placed in the well containing the anode and cathode. The semipermeable membrane was placed on top taking care not to trap bubbles of air underneath it. The top was screwed in position to hold the membrane in place. The platinum electrode was polarised at -0.6V with respect to the silver electrode. The current which flowed under these conditions was approximately 1 μA in stirred air-saturated water at 30°C. Air-saturated distilled water was added to the chamber and the sensitivity was adjusted to give a deflection of 96% of full scale deflection on the chart recorder. The recorder was set to this value since air saturated water contains 240 n mole oxygen $ml^{-1}$ and so 1 chart division was equivalent to 2.5 nmole oxygen $ml^{-1}$. The zero was set by the addition of a few crystals of sodium dithionite which rapidly absorbed the oxygen present in the water to become sodium dithionate and reducing the oxygen concentration to zero.

### 2.9.2 Preparation of Cell Suspension

Late exponential cultures were harvested by centrifugation at 13,500 g for 10min, washed in ice-cold 0.2M phosphate buffer pH 7.0 and resuspended in the same buffer. Organisms were starved for 3h at 30°C with air sparging through the culture to aerate

Figure 2.7 Schematic representation of an oxygen electrode.

A, Constant temperature water in; B, Water out; C, Cell chamber; D, Variable volume plunger with capilliary hole for injection into cell; E, Stirrer; F, Semi-permeable membrane; G, silver electrode; H, Platinum electrode; J, Saturated (3.8M) potassium chloride.


B
D
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A
F
H
J
E
G

the culture and metabolise residual intracellular carbon sources. 3ml of cell suspension was placed in the cell chamber and the basal rate (starvation rate) of oxygen uptake was measured. A fixed volume of carbon source was injected into the chamber (usually equivalent to 0.5 g carbon $l^{-1}$) and the rate of oxygen uptake measured. The actual rate of uptake due to the presence of the carbon compound was determined by subtracting the resting cell rate from the rate with a carbon source present. After each sample the electrode was washed several times with distilled water. The rate of oxygen uptake was expressed as µmole oxygen (mg dry wt)$^{-1}$ $h^{-1}$.

## 2.10 CHARACTERISATION OF THE POTENTIAL 3,6 DICHLOROPICOLINATE BREAKDOWN PRODUCT

### 2.10.1 Preparation of the Culture

35 µCi of 2, 6 [$^{14}$C] 3,6 dichloropicolinate was placed in a 200ml conical flask containing the defined medium supplemented with 4ml 10% (w/v) picolinate. The flask was inoculated with two 5ml aliquots of the mixed culture that had been stored with glycerol at -18°C (Section 2.2.1). The flask was shaken at 30°C and 50ml portions removed after 10, 20 and 30 days. These samples were centrifuged and the supernatant extracted with ether as described in section 2.6.4.

### 2.10.2 Thin Layer Chromatography

The ethereal solution was evaporated in a stream of dry air. The concentrated solution was spotted onto a thin layer chromatography plate (E. Merck, Darmstadt, Fed. Rep. Ger.). The plates were aluminium backed, with a coating of silica gel 60 without a fluorescent indicator. A chromatography tank was prepared by pouring in approximately 200ml of solvent (80 vol, ethyl acetate, 10 vol, methanol and 10 vol, glacial acetic acid) and left for 30 minutes in order to saturate the air with the solvent vapour. The spotted t.l.c. plate (sample and various standards) was placed in the tank and run until the solvent from was about 1 inch from the top of the plate. The spots on the dried plate were first visualised under u/v light at 360nm. The plates were also visualised in a tank containing iodine vapour. The spots were marked by pencil circles.

### 2.10.3 Analysis

The areas where compounds were visible under u/v light or iodine vapour were examined for radioactivity using a Geiger-Muller counter. Radioactive spots were scraped off into diethyl ether, methylated (Section 2.6.4) and analysed by gas chromatograhy.

## 2.11 MATERIALS

All materials were of Analar grade where possible. Picolinic acid was supplied by Aldrich Chemical Co. (Gillingham, Dorset, UK) 3,6-dichloropicolinic acid, analytical and technical grades, Methyl 3,6-dichloropicolinate, 3,6-dichloropyridinol, [$^{14}$C] 3,6-dichloropicolinic acid were kindly donated by The Dow Chemical Co., Kings Lynn, Norfolk, UK.

CHAPTER 3

# MINERALISATION OF 3,6 DICHLOROPICOLINIC ACID (36DCPA)

## 3.1 RESULTS

To test the feasibility of screening soil micro-organisms for any activity against recalcitrant molecules, the compounds first need to be tested for mineralisation in soil and an examination made for any metabolised breakdown products. It has been demonstrated that micro-organisms capable of the degradation of organic pollutants can be enriched for by application of biodegradable analogues to the microbial ecosystem (Focht & Alexander, 1970) and this phenomenon has been termed "analogue enrichment" (see section 5.1).

It is not necessary, when examining mineralisation alone, to use the high concentrations that are normally necessary for obtaining measurable growth in liquid media, so field application rates may be used. This gives a more accurate indication of the fate of the herbicide in the natural environment.

Four soils were used to study mineralisation. Two of these had shown high rates of 36DCPA disappearance in field trials (Dow Chemical Co., personal communication) and were thus termed high activity soils and the other two had shown long residence times of 36DCPA during field studies and so were termed low activity soils. All the soils were treated identically (see section 2.5) and were inoculated with 0.25 ppm ($\mu g\ g^{-1}$ soil) 2,6 [$^{14}$C]-3,6DCPA as the sole source of carbon.

Table 3.1 shows the amount of $^{14}CO_2$ absorbed from the soil culture effluent air, expressed as disintegration per minute, for each soil. Because the nature of the soils (i.e. their activity towards 36DCPA) was specified as high or low by the Dow Chemical Co., the times of sampling were varied for the two activities of soil.

# CHAPTER 3

## MINERALISATION OF 3,6 DICHLOROPICOLINIC ACID (36DCPA)

### 3.1 RESULTS

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Table 3.1 Amount of $^{14}CO_2$ absorbed by the absorbing reagent when field application rates of 2,6 [$^{14}$C]-36DCPA were to the soils specified. Values have been adjusted for blank and sterile control.

TABLE 3.1

| Time (days) | Activity of absorption reagent (D.P.M) | | | |
|---|---|---|---|---|
| | Soils | | | |
| | M46 | M47 | M51 | M53 |
| 1 | 6798 | ND | 8022 | ND |
| 2 | 12288 | ND | 11170 | ND |
| 3 | 19152 | ND | 25713 | ND |
| 4 | 33675 | ND | 36761 | ND |
| 5 | 56241 | ND | 48479 | ND |
| 7 | 104448 | ND | 71121 | ND |
| 10 | 268572 | 43956 | 144239 | 64026 |
| 14 | 357675 | ND | 181302 | ND |
| 21 | 644235 | 106743 | 310806 | 136242 |
| 28 | 941039 | ND | 491061 | ND |
| 30 | ND | 215558 | ND | 232221 |
| 42 | ND | 339876 | ND | 360179 |
| 55 | ND | 543484 | ND | 531408 |
| 67 | ND | 1113202 | ND | 1243759 |

Figure 3.1(a) shows the percentage of 36DCPA remaining in the soil at the various times tested. Control samples of heat sterilised soil plus identical quantities of 2,6 [$^{14}$C]-36DCPA and $CO_2$ absorption reagent plus scintillant were allowed for in the calculation of the percentage of 36DCPA remaining. After 28 days it can be seen that approximately 30% of the radio-active 36DCPA has disappeared from the culture vessel.

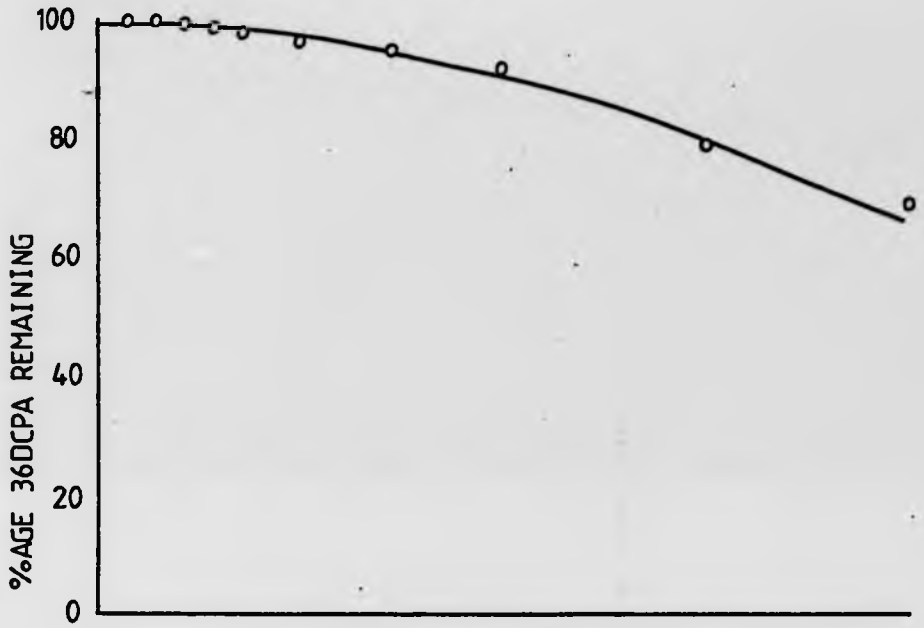
Figure 3.1(b) shows the disappearance of 36DCPA from the same soil but on a logarithmic scale. From this it can be seen that up to day 14 the rate of disappearance of 36DCPA was relatively slow (8% in 14 days). At day 14 there appeared to be a sharp rise in the degradation rate. The new rate of degradation was approximately 3 times that of the old rate. From this figure, the half life of 36DCPA in soil M46 was calculated to be 43 days (calculated from the steeper half of the graph).

Figures 3.2(a) and (b), 3.3(a) and (b) and figures 3.4(a) and (b) show similar results for soils M51, M47 and M53 respectively. Soils M47 and M53 were the biologically less active soils. The half lives have been calculated to be 71 days, 181 days and 152 days respectively (calculated from the shallower portion of the graph). In soil M51 there appeared to be the same increase in the rate of degradation that was apparent in soil M46 but it appeared to be absent in the two other soils. This shows, to some extent, that the larger organic component of these soils was more able to produce a more rapid mechanism of degradation after a suitable induction period.

## 3.2 DISCUSSION

Johnen & Drew (1977) suggest that "any system for evaluating pesticidal effects must reflect the situation that occurs in the natural environment," and this can also be extended to any study of microbial degradation of the said pesticide. Laboratory tests involving chemical concentrations greater than those in nature may not correctly assess the rate of biodegradation in natural ecosystems.

Figure 3.1 (a) Disappearance of 2,6 [$^{14}$C]-36DCPA in soil M46 (a) linear, (b) logarithmic.


100
80
60
40
20
0
%AGE 36DCPA REMAINING

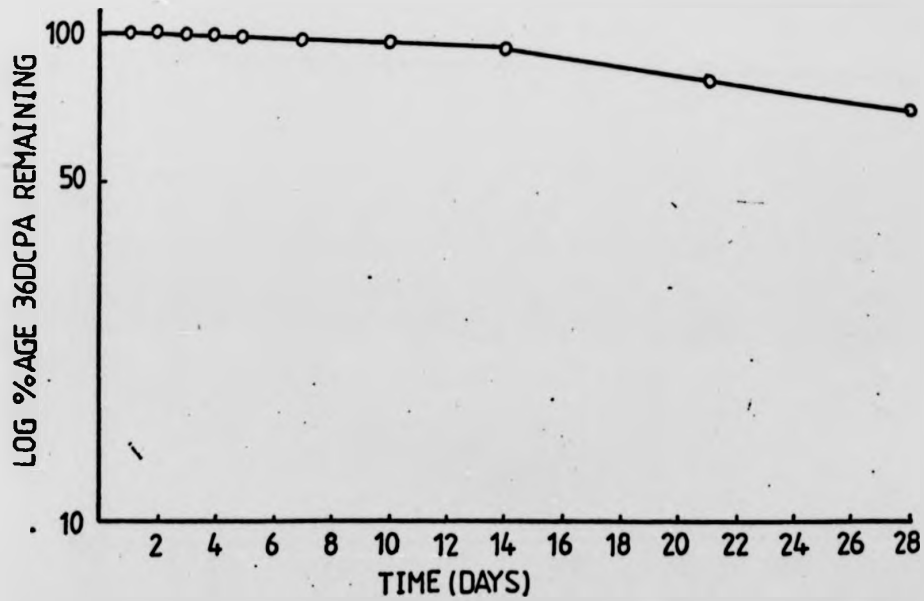

100
50
10
LOG %AGE 36DCPA REMAINING
2
4
6
8
10
12
14
16
18
20
22
24
26
28
TIME (DAYS)

Figure 3.2 Disappearance of 2,6 $^{14}$C-36DCPA from soil M51 (a) linear (b) logarithmic.

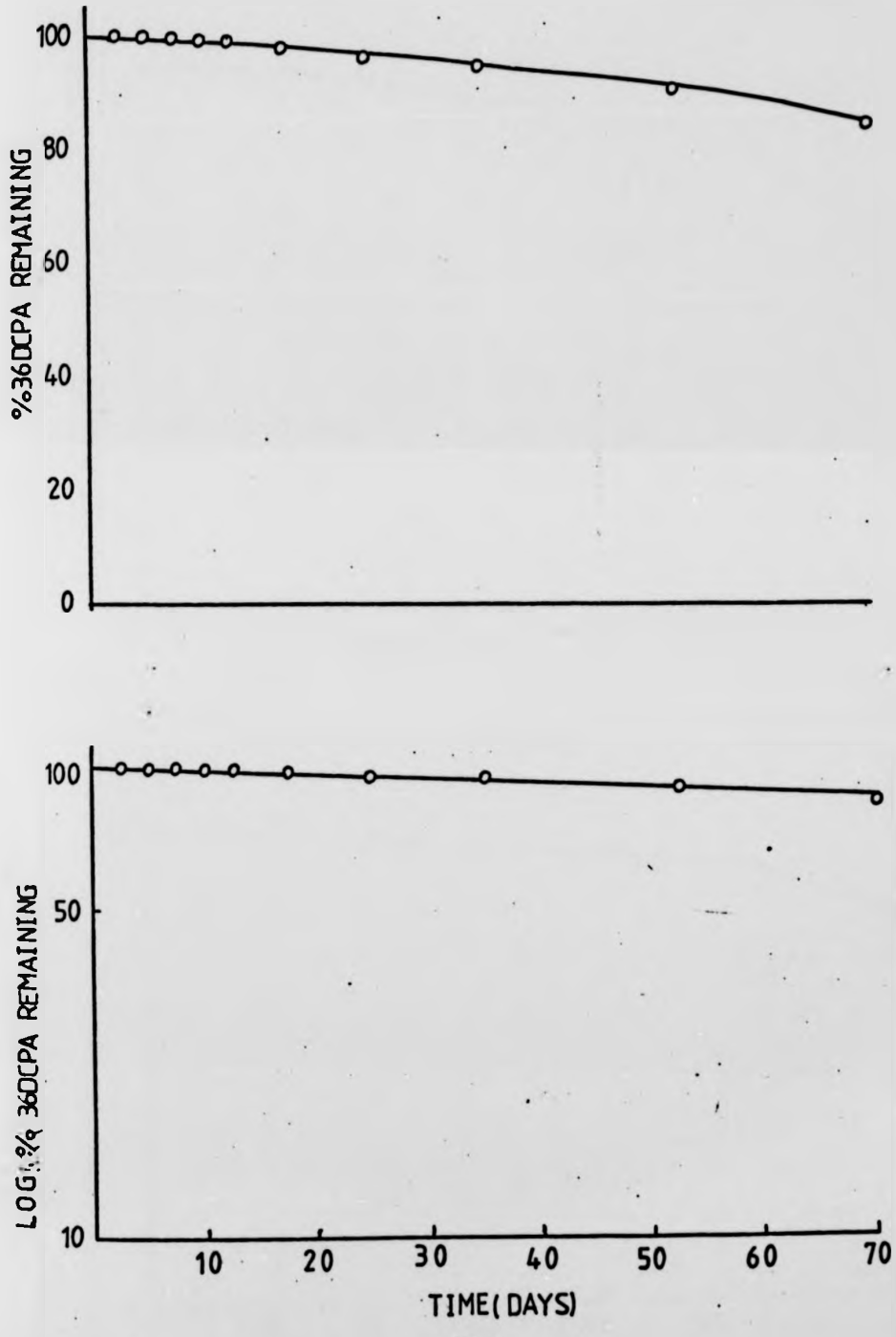
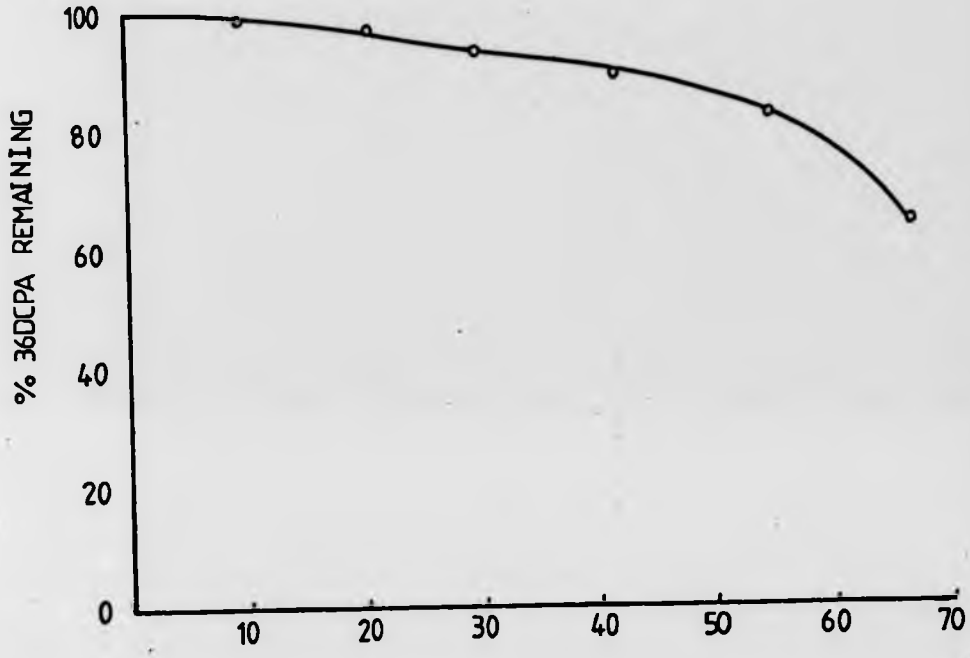

100
80
60
40
20
0
%36DCPA REMAINING
100
50
10
LOG%36DCPA REMAINING
10
20
30
40
50
60
70
TIME(DAYS)

Figure 3.3 Disappearance of 2,6 $^{14}$C-36DCPA from soil M47 (a) linear (b) logarithmic.


100
80
60
40
20
0
% 36DCPA REMAINING
10
20
30
40
50
60
70

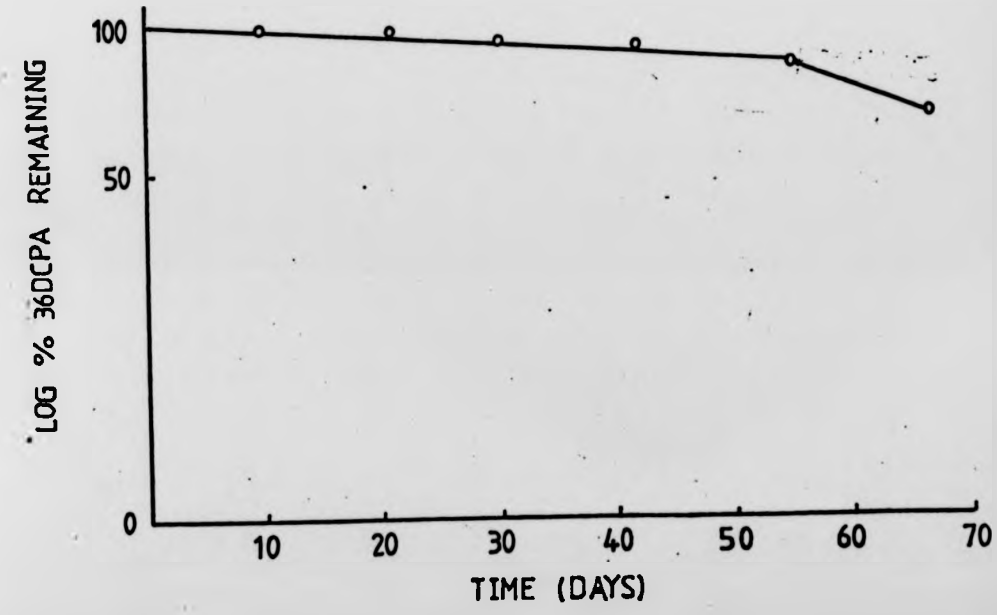
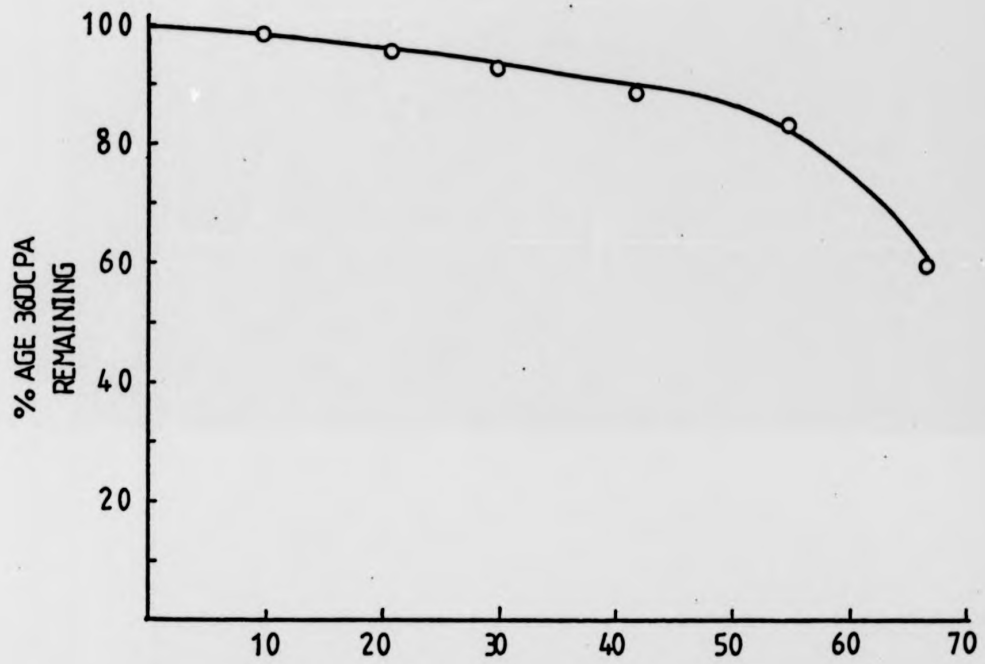
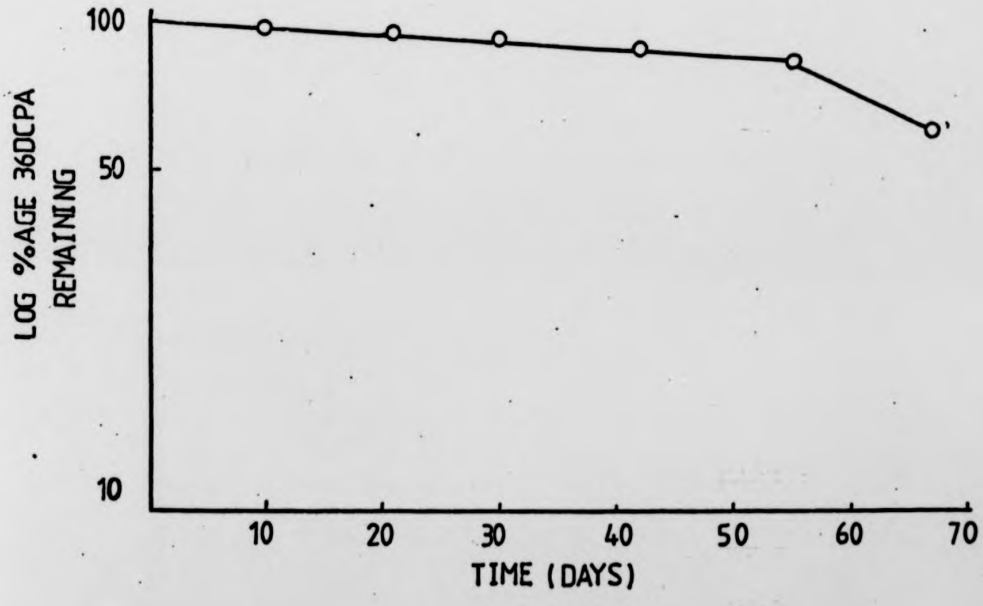

100
50
0
LOG % 36DCPA REMAINING
10
20
30
40
50
60
70
TIME (DAYS)

Figure 3.4 Disappearance of 2,6 $^{14}$C-36DCPA from soil M53 (a) linear (b) logarithmic.


100
80
60
40
20
% AGE 36DCPA REMAINING
10
20
30
40
50
60
70


100
50
10
LOG %AGE 36DCPA REMAINING
10
20
30
40
50
60
70
TIME (DAYS)

However, using the low concentrations of 36DCPA that are applied in an agricultural environment it is not possible to measure microbial growth by any standard technique. This experiment was a useful guide to the feasability of degradation studies since it showed that 36DCPA did breakdown in soil to carbon dioxide and other unidentified metabolites. Pik, Peake, Strosher and Hodgson (1977) have examined the fate of the 3,6-dichloropicolinic acid in soils in Canada and have concluded that "no metabolites accumulate during the degradative process so that no additional contamination of the environment occurs."

The results given in this section do, to a certain extent, agree with this finding since both of the [$^{14}$C]-molecules are in the ring of the molecule. In order to release $^{14}CO_2$ the ring must be cleaved and aliphatic chlorocarbons are considered to be less recalcitrant than aromatic chlorocarbons.

Pik et al (1977) also concluded that "the degradation that occurs is... inversely correlated to the organic matter content of the soil" and in this matter the findings shown are completely opposite. In the more organic soils the half life was approximately one third the half life of the more inorganic soils. Cheng, Fuhr, Jarczyk & Mittelstaedt (1978) studied the degradation of methabenzthiazuron is the soil by comparing the rates of $^{14}CO_2$ evolution from the soil and found that the more organic soil of the two studied was more active against the herbicide. Although a different compound was used these findings do support the results presented here.

The two rates of degradation may be explained by the component populations in the culture vessel adapting to the herbicide and producing more abundant populations that were able to attack the herbicide, or by producing a more varied population by the evolution of some of the component populations.

Because 36DCPA did break down in soil it was considered that further studies to find a microbial population or mixed microbial population that was able to utilise 36DCPA as sole source of carbon and energy was justified.

# CHAPTER 4

## ENRICHMENT EXPERIMENTS

### 4.1 RESULTS

By studying the response of the complete microbiota to selected environmental treatments in the laboratory, the action of these treatments in the natural environment is more closely mirrored, than by studying monocultures of micro-organisms. The enrichment experiments performed were designed to select for a microbial population, perhaps containing different species, which could act upon, chemically alter, degrade, or use as a source of carbon and/or energy, the herbicide 3,6-dichloropicolinate (36DCPA).

Two methods of continuous-flow enrichment and selection, the soil columns and the chemostat, were used to isolate populations which could degrade 36DCPA. Direct enrichments, in shake flasks were also used but these proved to be of little value in selecting for the desired capabilities.

#### 4.1.1 Continuous-Flow Soil Columns

Soil columns were set up and sampled as described in section 2.4.2. A peaty loam soil, supplied by The Dow Chemical Co., and a clay soil collected from Warwick University campus were mixed and prepared as detailed in section 2.4.2.1. From preliminary closed culture experiments it was observed that utilisation of the nitrogen molecule in 36DCPA might be possible. In order to test this hypothesis two soil columns were set up. The first was supplied with 36DCPA as the sole source of carbon and the second was supplied with 36DCPA as the sole source of nitrogen and benzoate as the carbon source.

Initially direct continuous enrichment was studied, supplying the columns with only 36DCPA. This proved to be of little value in selecting for any species which had the desired capabilities, similar to direct enrichment. The absorbance of the effluent culture was never greater than 0.05 and the maximum viable count obtained was $4.7 \times 10^6$ organisms $ml^{-1}$.

Due to the much reduced viable counts obtained by the direct continuous-flow enrichment a subsidiary carbon source was added to the influent media. Benzoate was chosen for this role since, like 36DCPA, it was aromatic and acidic in nature, but unlike 36DCPA, it was known to be readily assimilated by soil micro organisms. The benzoate in the medium increased the viable count and thus exposed more organisms to 36DCPA, increasing the probability of obtaining a micro-organism that could utilise 36DCPA.

Fresh soil was added to the two columns, described earlier, and they were designated column A and column B. Column A was supplied with 36DCPA as sole source of nitrogen, at a concentration of 0.11g nitrogen $l^{-1}$, and benzoate as the sole source of carbon, at a concentration of 0.5g carbon $l^{-1}$. Column B was supplied with ammonium sulphate as the source of nitrogen (0.11g nitrogen $l^{-1}$) and 36DCPA and benzoate as the sources of carbon (0.5g carbon $l^{-1}$ total, in the ratio 2:1). The columns were sampled every 3-4 days initially and subsequently every 7-8 days for a total of 180 days. During the middle of the sampling period, day 60 to day 100 the columns were left operating without sampling. The parameters measured were absorbance at 600nm, concentration of free chloride ions in solution, and the growth of the organisms in the effluent media on agar plates. The carbon and nitrogen sources on the plates were, 36DCPA as carbon and nitrogen source, 36DCPA as carbon and ammonium ions as nitrogen, benzoate as carbon and 36DCPA as nitrogen, glycerol as carbon and 36DCPA as nitrogen, picolinate as carbon and nitrogen sources, and nutrient agar to give an estimation of total viable count.

4.1.1.1 Column A

Figure 4.1 shows the absorbance and free chloride ion concentration of the effluent from column A. It is possible that the 36DCPA, present as a nitrogen source, did act as a secondary source of carbon. The correlation between the absorbance (where this was in some manner directly proportional to biomass) and the chloride ion concentration was calculated using the following formula:

Correlation coefficient = r

$$r = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\left[\sum x^2 - \frac{(\sum x)^2}{n}\right]\left[\sum y^2 - \frac{(\sum y)^2}{n}\right]} \qquad 4.1$$

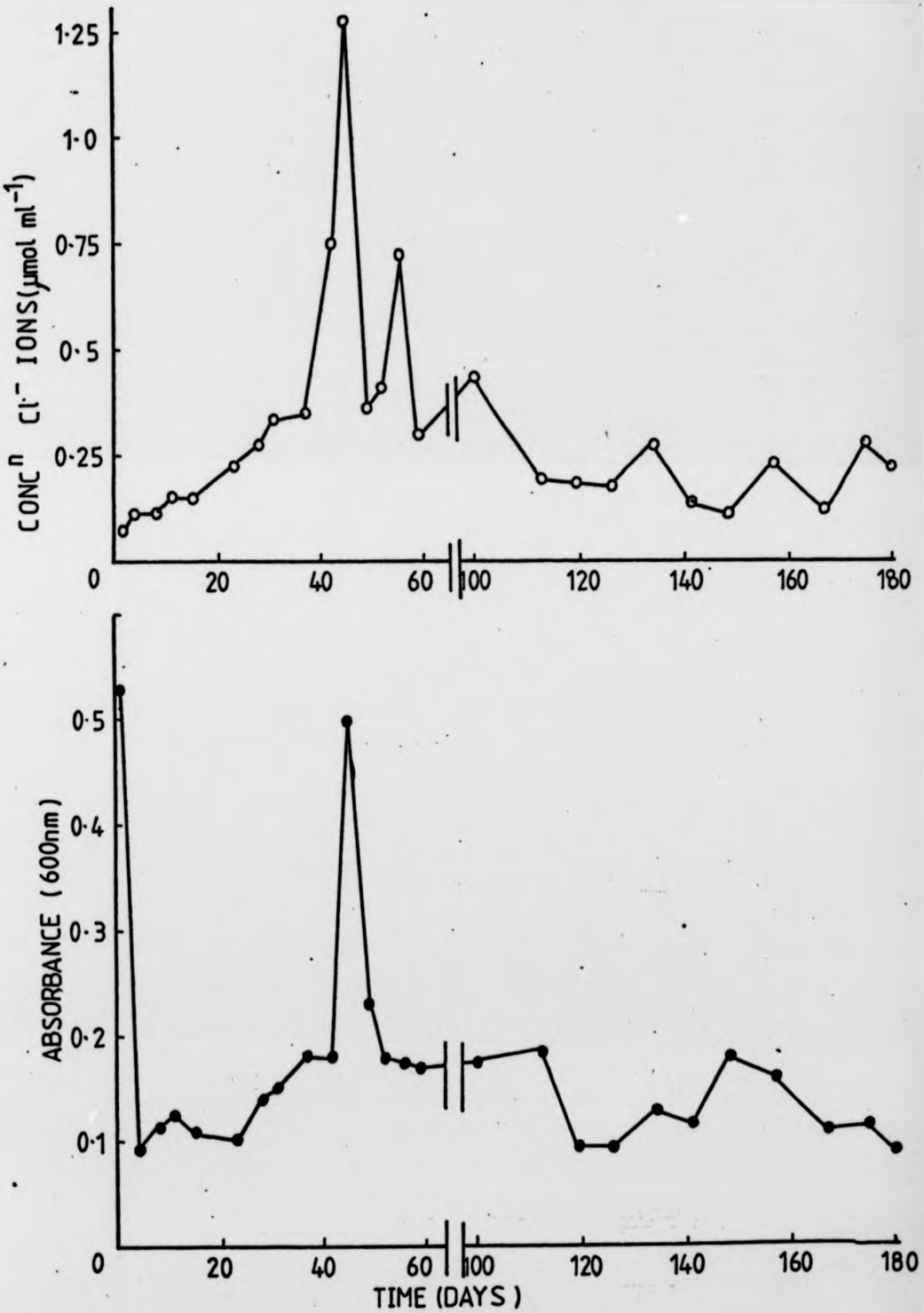
where n = number of points
x = values of absorbance
y = values of chloride ion concentration.

In this case r = 0.316. It is clear that there was some relationship between the concentration of chloride ions and the biomass produced in the column, as expressed in terms of absorbance. Indeed, to begin with, the slow rise in absorbance was almost mirrored by a rise in free chloride ions. At the peak of absorbance (day 45) there was a similar peak in free chloride ions. This appeared to indicate that the 36DCPA was being degraded by the action of the micro-organisms in the soil column, since 36DCPA was the only molecule with chloro groups present in the medium.

The maximum amount of chloride ion release possible was 15.72 $\mu mol\ ml^{-1}$ (calculated on the amount of 36DCPA added).

The maximum amount of chloride ions actually released was 1.25 $\mu mol\ ml^{-1}$ and the average value was approximately 0.3 $\mu mol\ ml^{-1}$. 1 $\mu mol\ ml^{-1}$ of chloride release corresponds to 3% of the 36DCPA added totally mineralised, that is, 0.040g $36DCPA^{-1}$. The very

Figure 4.1 Absorbance at 600nm (●) and concentration of free chloride ions (O) from the effluent of column A.


1·25
1·0
0·75
0·5
0·25
0
CONC$^{n}$ $Cl^{-}$ IONS(µmol $ml^{-1}$)
20
40
60
100
120
140
160
180
0·5
0·4
0·3
0·2
0·1
0
ABSORBANCE (600nm)
TIME (DAYS)

low concentration of chloride ions released, however, suggested in agreement with the results described in section 3.1, that the breakdown and release of chloride ions from 36DCPA occurred at a very slow rate.

Figure 4.2 shows the viable count of organisms, in the column effluent, growing on nutrient agar. During the early period of perfusion of the column the viable count oscillated markedly, but towards the latter period, the count became much more stable, levelling off at approximately $1.3 \times 10^8$ viable organisms $ml^{-1}$.

Figures 4.3 and 4.4 show the growth of organisms, in the column effluent, on agar containing 36DCPA as the sole source of carbon and nitrogen (concentration, 0.11g nitrogen $l^{-1}$) and 36DCPA as the sole source of carbon (concentration, 0.5g carbon $l^{-1}$) with ammonium ions as a source of nitrogen respectively. The counts $ml^{-1}$ oscillated throughout the entire time. The colonies that were observed as this type of agar were very small (<0.5mm diameter), mainly translucent and cream or colourless. All attempts at transferring the colonies to a second plate containing 36DCPA alone, or to liquid culture containing 36DCPA alone, failed. Both figures show a decrease in the number of organisms capable of growing on the agar, over the time period. If any of these organisms had been able to utilise 36DCPA in any manner, it would be expected that the number of viable organisms in the soil columns would have increased, rather than decreased.

Figures 4.5 and 4.6 show the count of viable organisms on plates containing 36DCPA as the sole source of nitrogen (0.11g nitrogen $l^{-1}$) and benzoate and glycerol, respectively, as the major source of carbon (0.5g carbon $l^{-1}$). There was very little difference between the populations in the soil that could grow on benzoate (figure 4.5) or glycerol (figure 4.6). Many of these colonies were replica plated onto agar containing 36DCPA alone to establish whether they possessed activity against 36DCPA when present as the carbon and nitrogen source. Some colonies were, also, inoculated into liquid medium containing 36DCPA as the sole source of carbon and nitrogen (0.11g nitrogen $l^{-1}$) but these all failed to show activity against 36DCPA when present alone (as indicated by the release of chloride ions).

Figure 4.2 Viable count of organisms, growing on nutrient agar, from the effluent of soil column A.

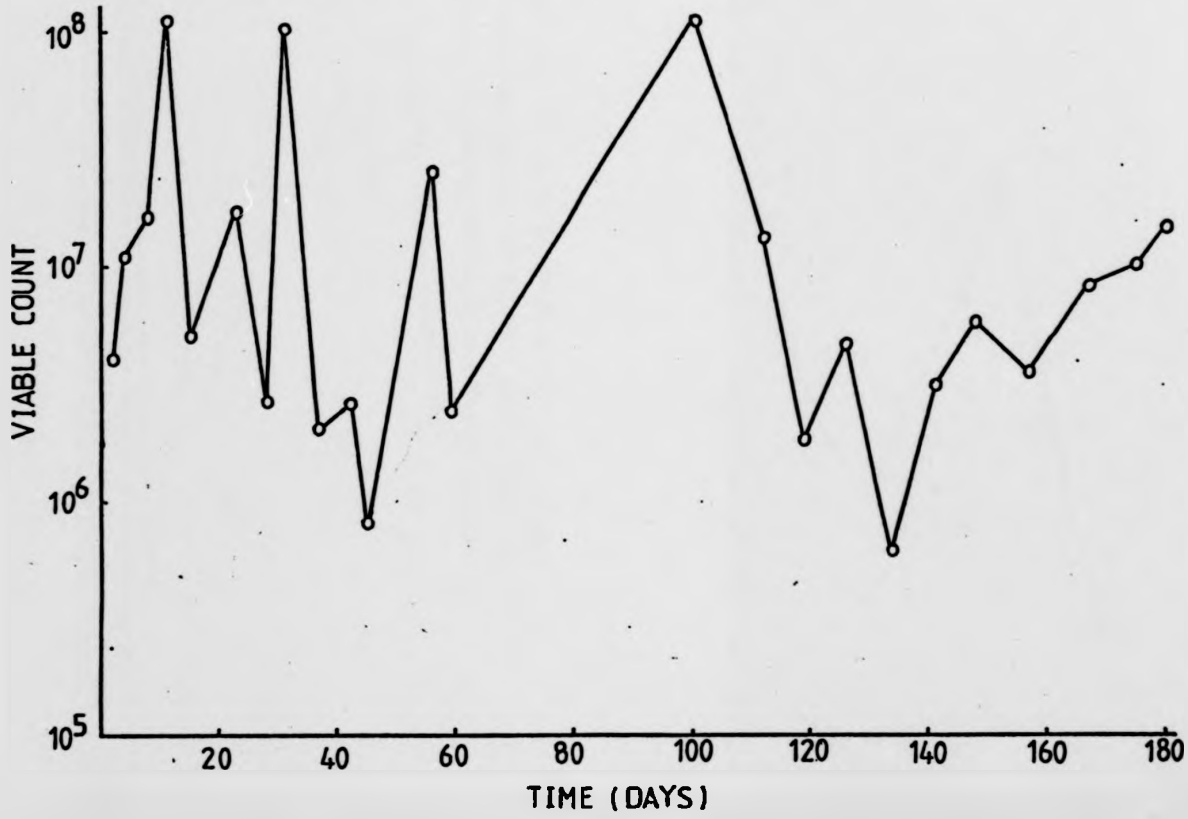
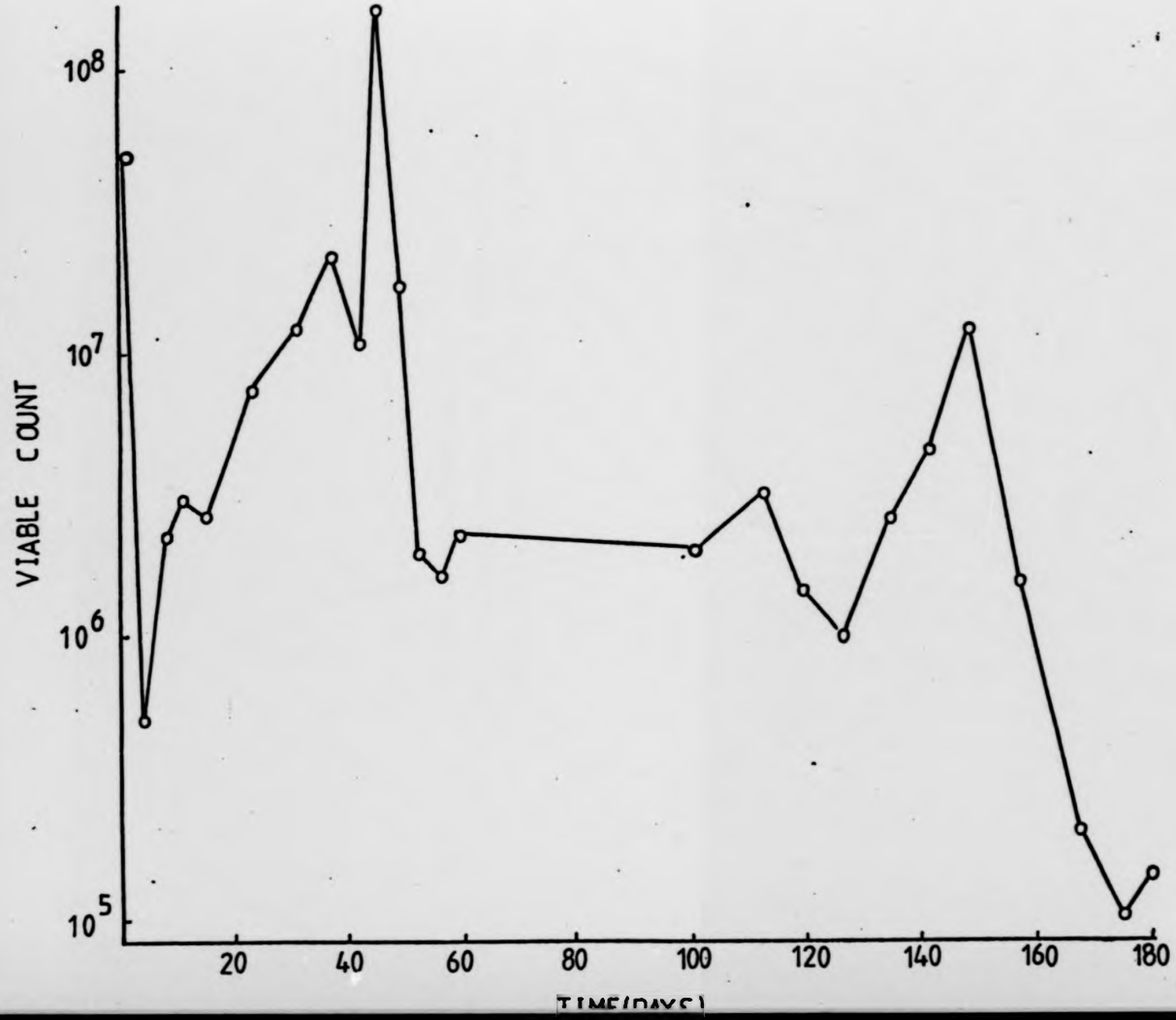

VIABLE COUNT
$10^8$
$10^7$
$10^6$
$10^5$
20
40
60
80
100
120
140
160
180
TIME (DAYS)

Figure 4.3 Viable count of organisms, growing on agar containing 36DCPA as carbon and nitrogen sources, from the effluent of column A.


VIABLE COUNT
$10^8$
$10^7$
$10^6$
$10^5$
20
40
60
80
100
120
140
160
180
TIME(DAYS)

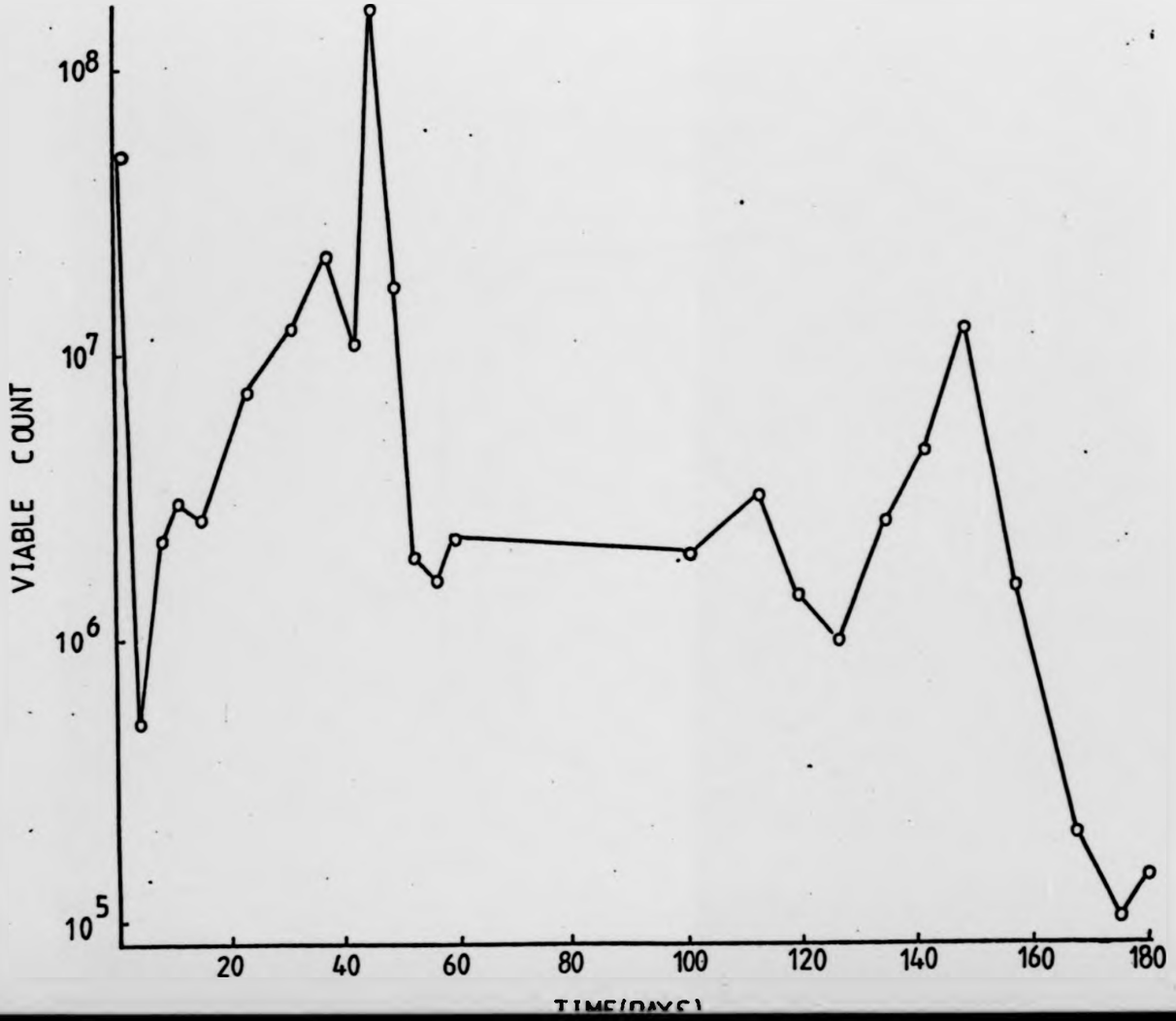

VIABLE COUNT
$10^8$
$10^7$
$10^6$
$10^5$
20
40
60
80
100
120
140
160
180
TIME(DAYS)


Figure 4.4 Viable count of organisms, growing on agar containing 36DCPA as sole source of carbon, from the effluent of column A.

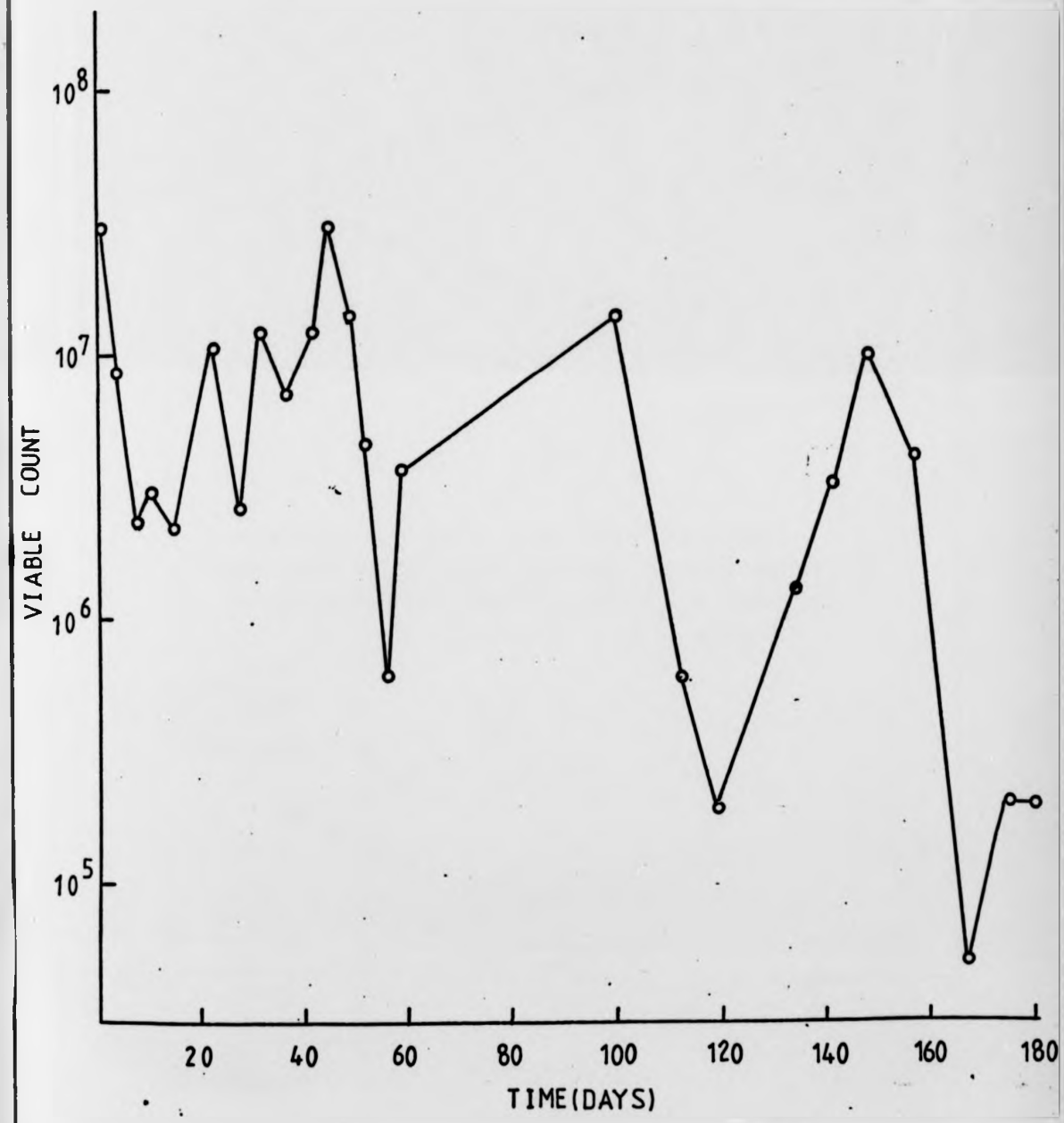

VIABLE COUNT
$10^8$
$10^7$
$10^6$
$10^5$
20
40
60
80
100
120
140
160
180
TIME(DAYS)

Figure 4.5 Viable count of organisms, growing on agar containing glycerol as the carbon source and 36DCPA as the nitrogen source, from the effluent of column A.

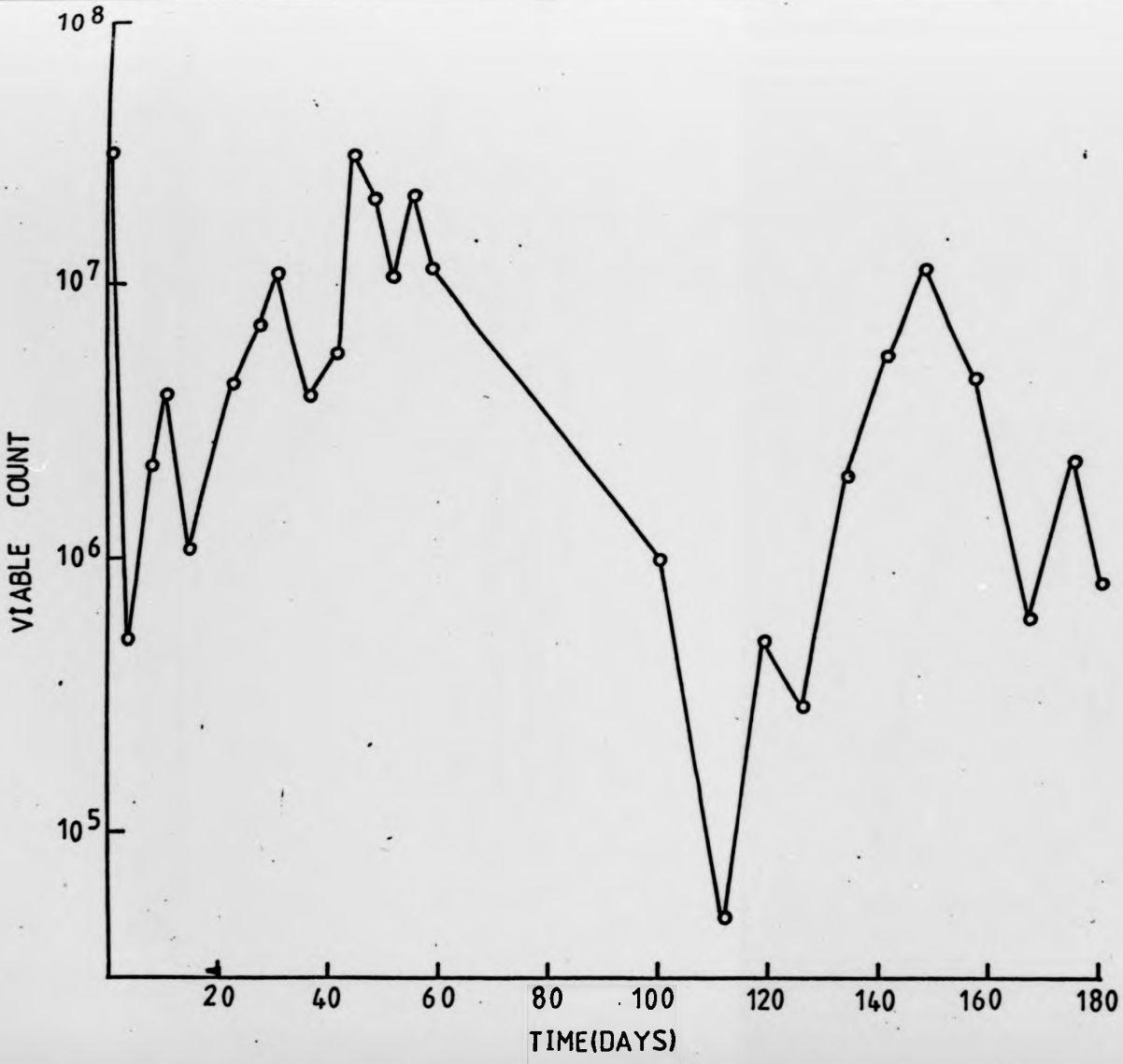

$10^8$
$10^7$
$10^6$
$10^5$
VIABLE COUNT
20
40
60
80
100
120
140
160
180
TIME(DAYS)

Figure 4.6 Viable count of organisms, growing on agar containing benzoate as the carbon source and 36DCPA as the nitrogen source, from the effluent of column A.

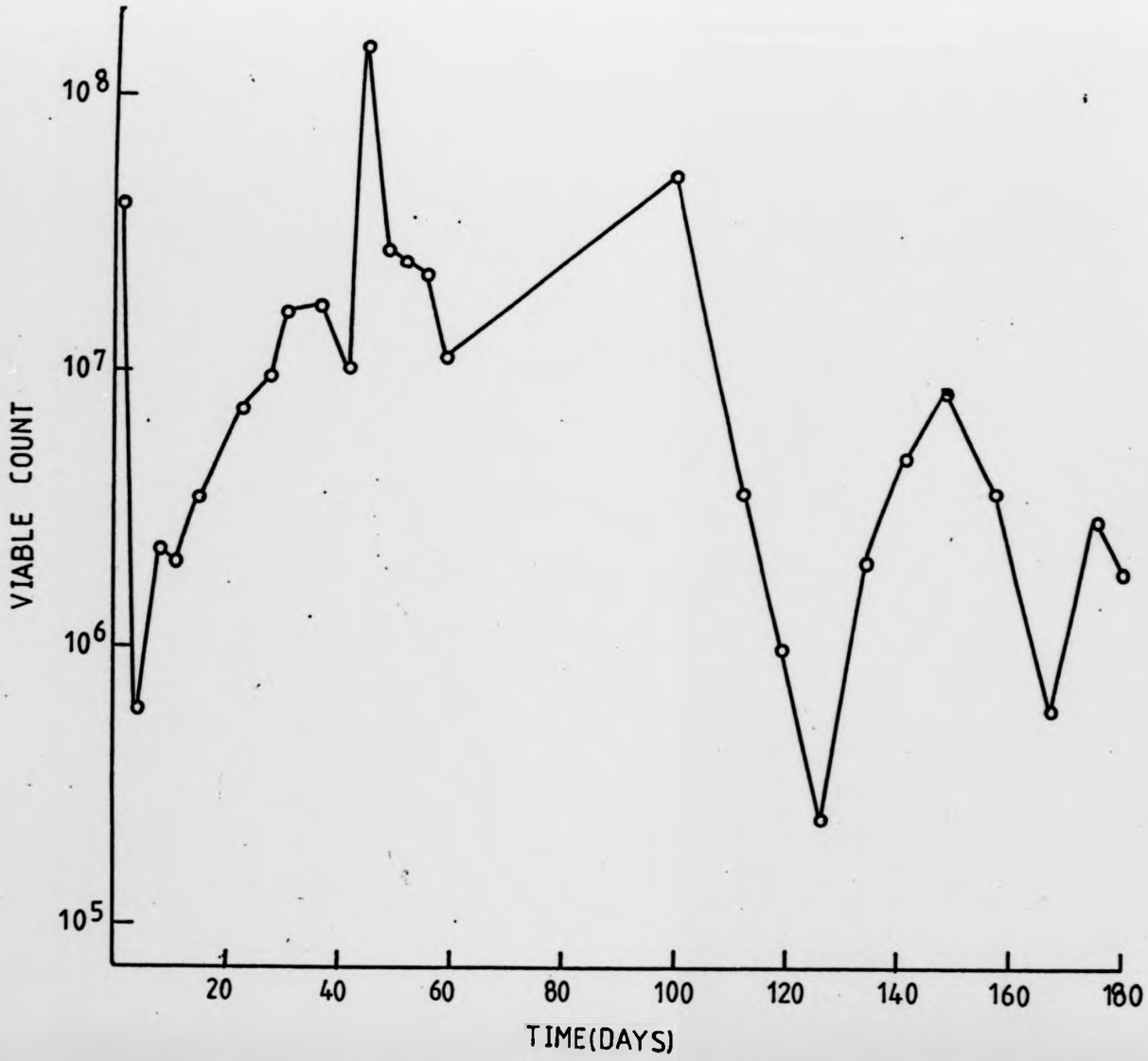

VIABLE COUNT
$10^8$
$10^7$
$10^6$
$10^5$
20
40
60
80
100
120
140
160
180
TIME(DAYS)

Figure 4.7 shows the viable count of organisms on agar containing picolinate as the sole source of carbon and nitrogen (concentration, 0.11g nitrogen $l^{-1}$). There was an approximate ten fold decrease in the viable count on picolinate agar, compared with all other types of agar previously mentioned. The colonies were, in general, much larger than those growing on agar containing 36DCPA alone (figure 4.3 and 4.4).

4.1.1.2 Column B

Figure 4.8 shows the absorbance at 600nm and the concentration of free chloride ions from column B. The correlation between the absorbance and free chloride concentration was calculated using the same equation as that used in section 4.1.1.1. In this case $r = 0.2197$ showing some correlation between these two parameters. The actual concentration of free chloride ions released was approximately 0.15 $\mu$mol $ml^{-1}$, about half of that released from 36DCPA in column A. The maximum amount of free chloride possible was 9.26 $\mu$mol $ml^{-1}$. The average absorbance for column B was approximately 0.2.

Figure 4.9 shows the viable count of organisms in the column effluent, grown on nutrient agar. The number of viable organisms $ml^{-1}$ began to achieve a constant level at approximately $6.5 \times 10^7$ organisms $ml^{-1}$ but suddenly the count dropped to $2 \times 10^5$ organisms $ml^{-1}$ on day 175 and continued to fall on day 180.

Figures 4.10 and 4.11 show the growth of organisms from the effluent of column B on agar containing 36DCPA alone, as the carbon and nitrogen source (concentration, 0.11g nitrogen $l^{-1}$, Figure 4.10) and as the carbon source (concentration, 0.5g carbon $l^{-1}$) with ammonium ions as the nitrogen source (figure 4.11). There was no general trend in the count of viable organisms $ml^{-1}$ on this agar. The colonies were replica plated, and subcultured into liquid media, in a similar manner to that described for column A, but all of the organisms failed to grow, or exhibit the release of chloride ions into the supernatant.

Figure 4.7 Viable count of organisms, growing on agar containing picolinate as the sole source of carbon and nitrogen, from the effluent of column A.

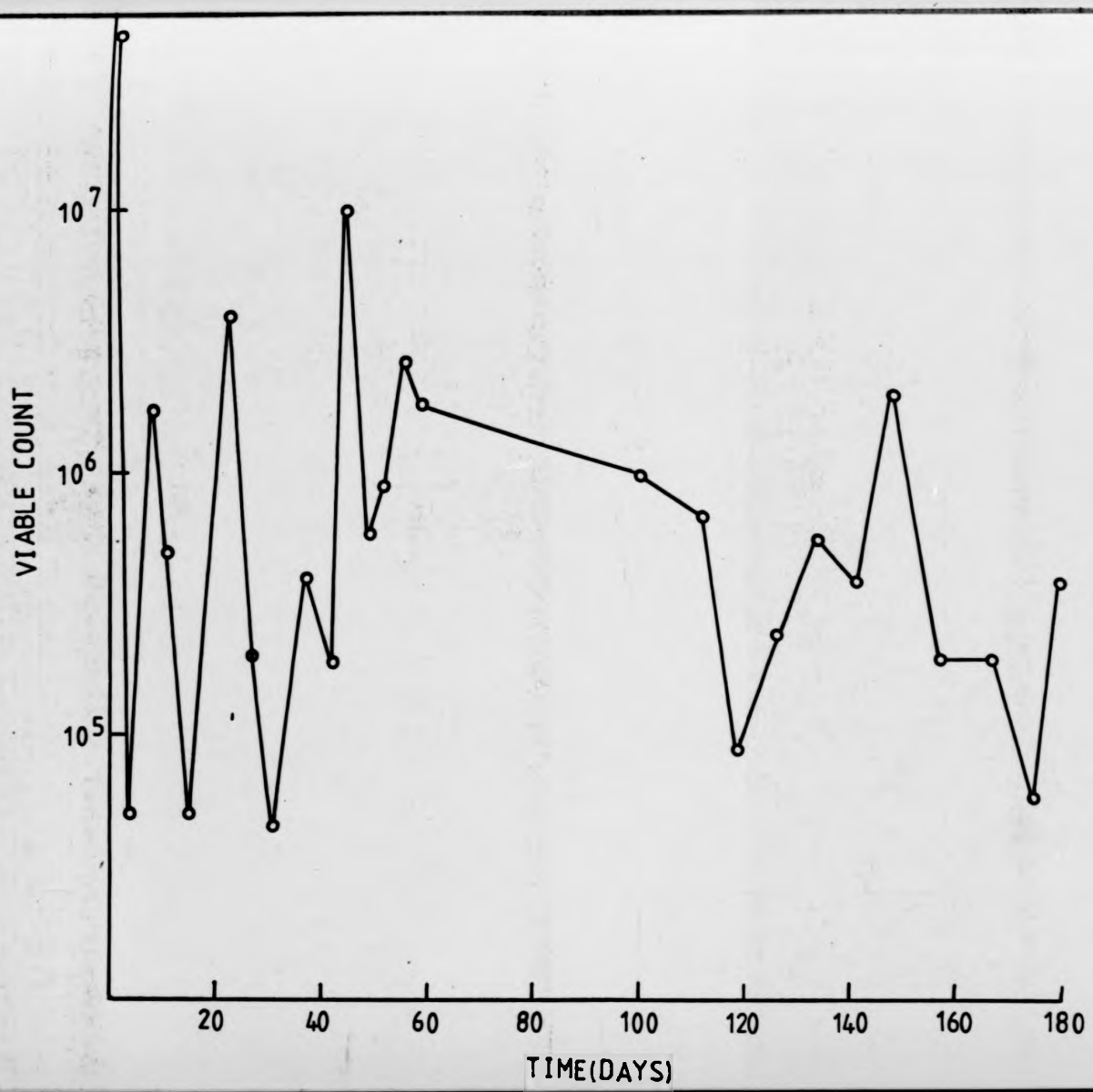

VIABLE COUNT
$10^7$
$10^6$
$10^5$
20
40
60
80
100
120
140
160
180
TIME(DAYS)

Figure 4.8 Absorbance at 600nm (●) and concentration of free chloride ions (○) from the effluent of column B.

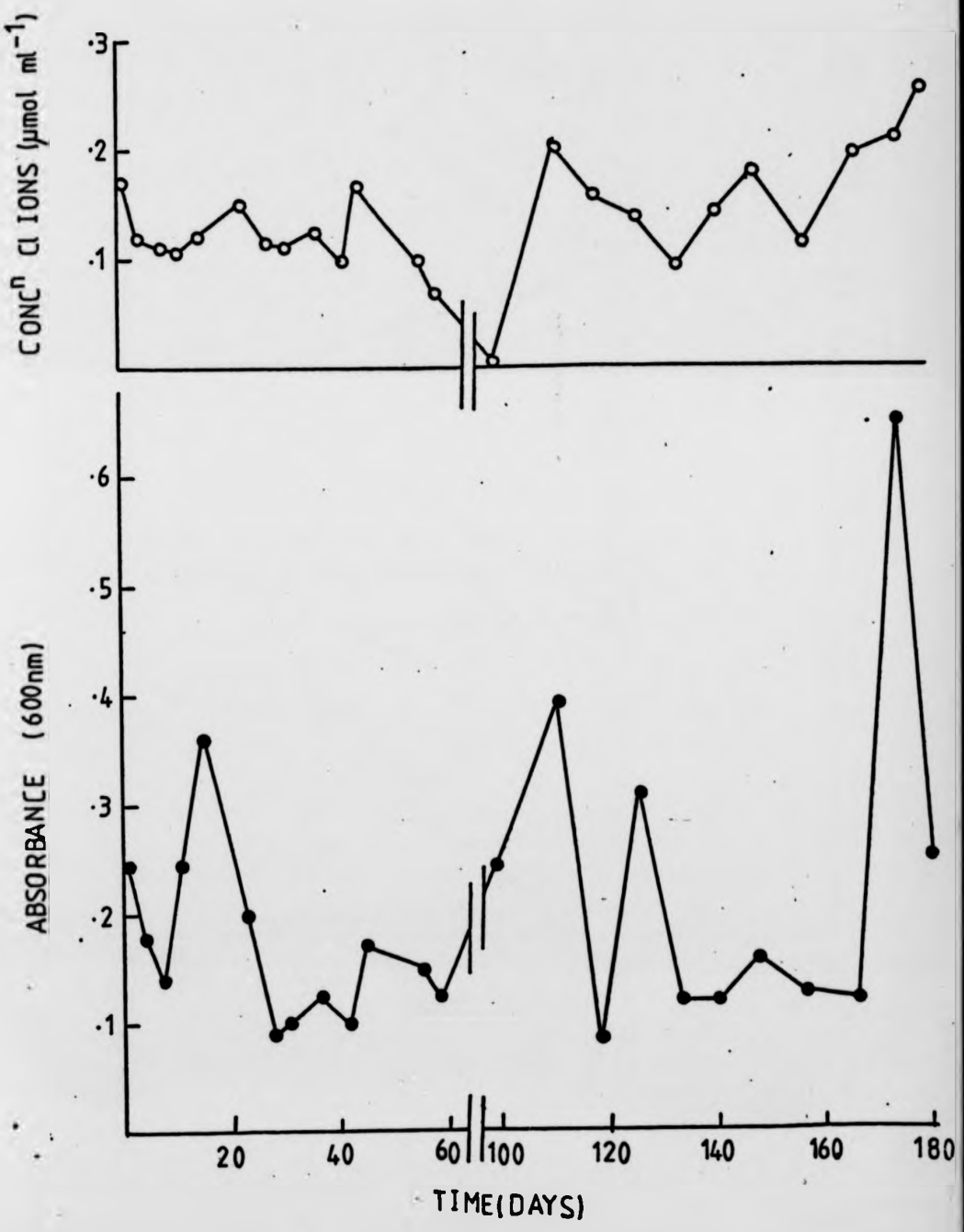

CONC$^{n}$ Cl IONS (µmol ml$^{-1}$)
·3
·2
·1
ABSORBANCE (600nm)
·6
·5
·4
·3
·2
·1
20
40
60
100
120
140
160
180
TIME(DAYS)

Figure 4.9 Viable count of organisms, growing on nutrient agar, from the effluent of column B.

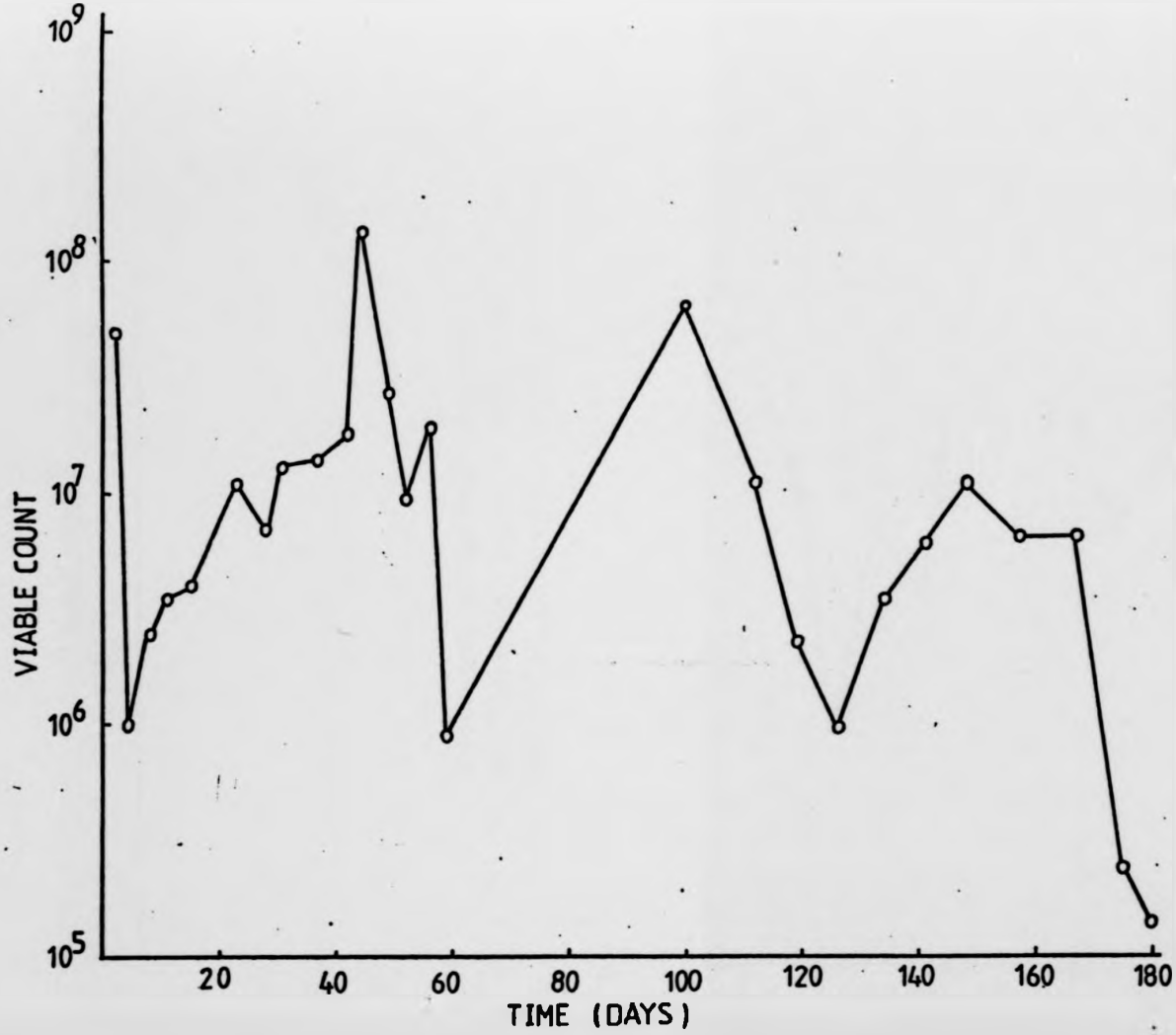

VIABLE COUNT
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
20
40
60
80
100
120
140
160
180
TIME (DAYS)

Figure 4.10 Viable count of organisms, growing on agar containing 36DCPA as carbon and nitrogen sources, from the effluent of column B.

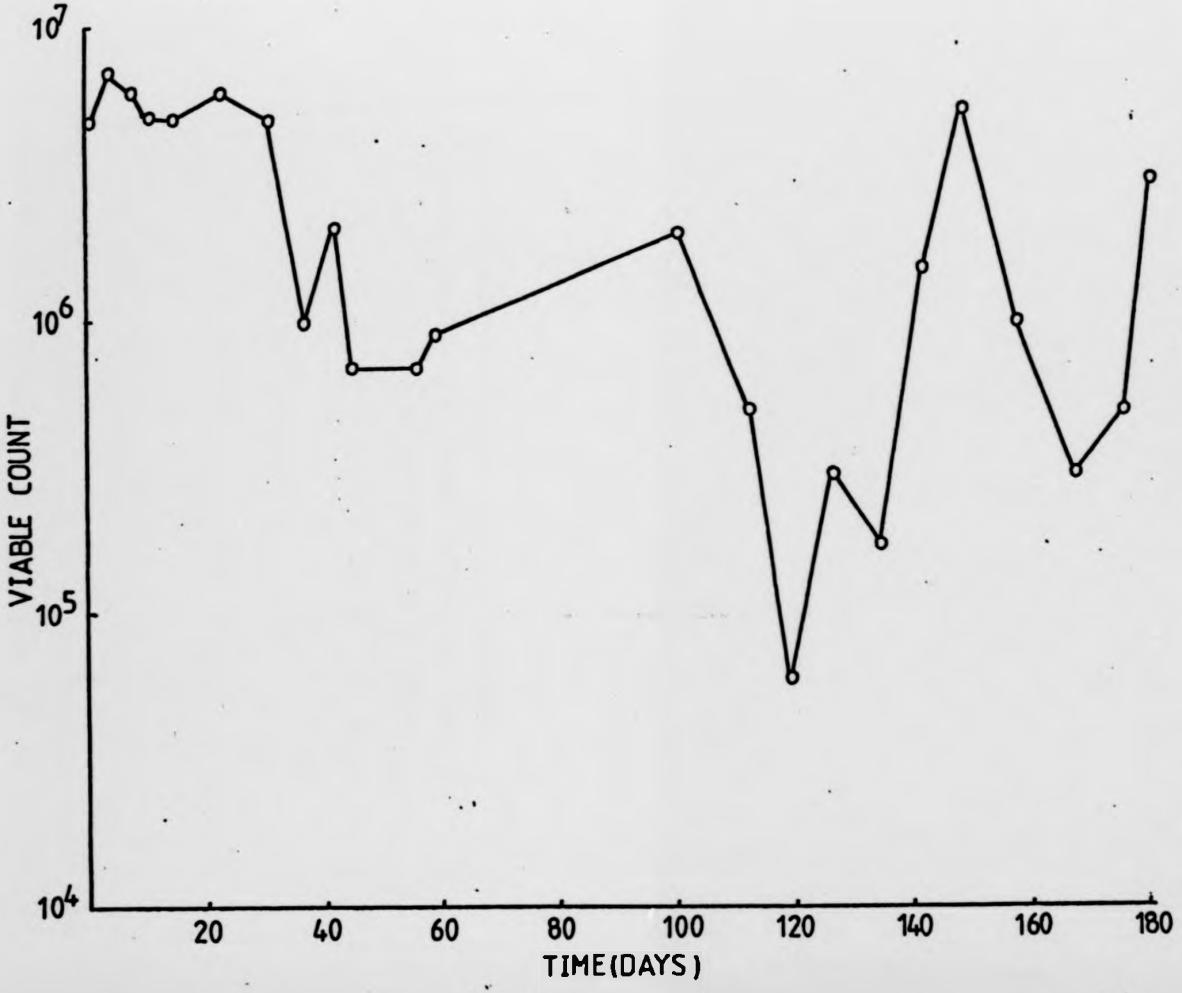

$10^7$
$10^6$
$10^5$
$10^4$
VIABLE COUNT
20
40
60
80
100
120
140
160
180
TIME(DAYS)


Figure 4.11 Viable count of organism, growing on agar containing 36DCPA as the sole source of carbon, from the effluent of column B.

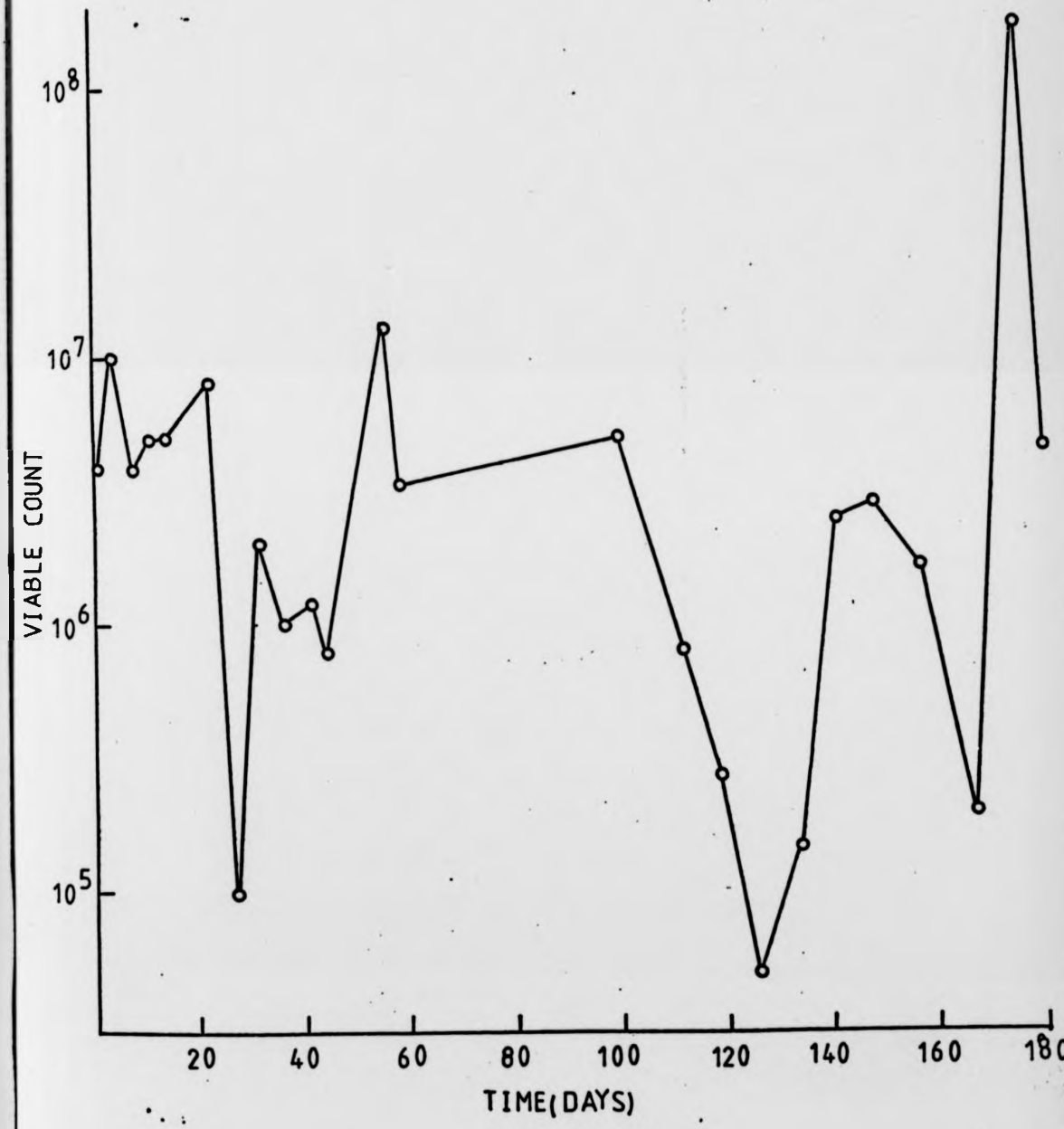

VIABLE COUNT
$10^8$
$10^7$
$10^6$
$10^5$
20
40
60
80
100
120
140
160
180
TIME(DAYS)

Figure 4.12 and 4.13 show the growth of viable organisms from the effluent of column B on agar containing 36DCPA as the source of nitrogen (concentration 0.11 nitrogen $l^{-1}$) and benzoate and glycerol, respectively, as the source of carbon (concentration 0.5g carbon $l^{-1}$). All the colonies that were plated onto agar containing 36DCPA alone, or inoculated into media containing 36DCPA alone, failed to exhibit any of the desired capabilities. There was no release of chloride ions into the solution. There was no general trend in the viable counts and, again, the oscillation of the counts tended to dampen towards the end of the experiment.

Figure 4.14 shows the viable organisms from the effluent of column B growing on agar containing picolinate as the sole source of carbon and nitrogen (concentration, 0.11g nitrogen $l^{-1}$). Towards the later period of time the viable count appeared to be increasing to a level similar to that of the other plates (figures 4.8 to 4.13). Like column A, however, there was an approximate ten fold decrease in the number of variable organsims $ml^{-1}$ that were capable of growing on this type of agar.

### 4.1.2 Chemostat Enrichment

Two types of chemostat enrichment were used to select for micro-organisms which were able to degrade 36DCPA. The first enrichment procedure was an adaption of a microbial population growing on picolinate, to grow on 36DCPA. This will be described in more detail in Chapter 5.

The second enrichment procedure was to establish organisms that were able to utilise 36DCPA as the sole source of carbon. The type of fermenter used in this enrichment experiment was the LHE series 500 fermenter. The influent medium was minimal medium, initially containing glycerol, but, later, benzoate as the major source of carbon (concentration 0.5g carbon $l^{-1}$) and supplemented with 36DCPA as the subsidiary source of carbon (concentration 0.1g carbon $l^{-1}$) and the temperature was maintained at 30°C.

Figure 4.12 Viable count of organisms, growing on agar containing glycerol as the carbon source and 36DCPA as the nitrogen source, from the effluent of column B.

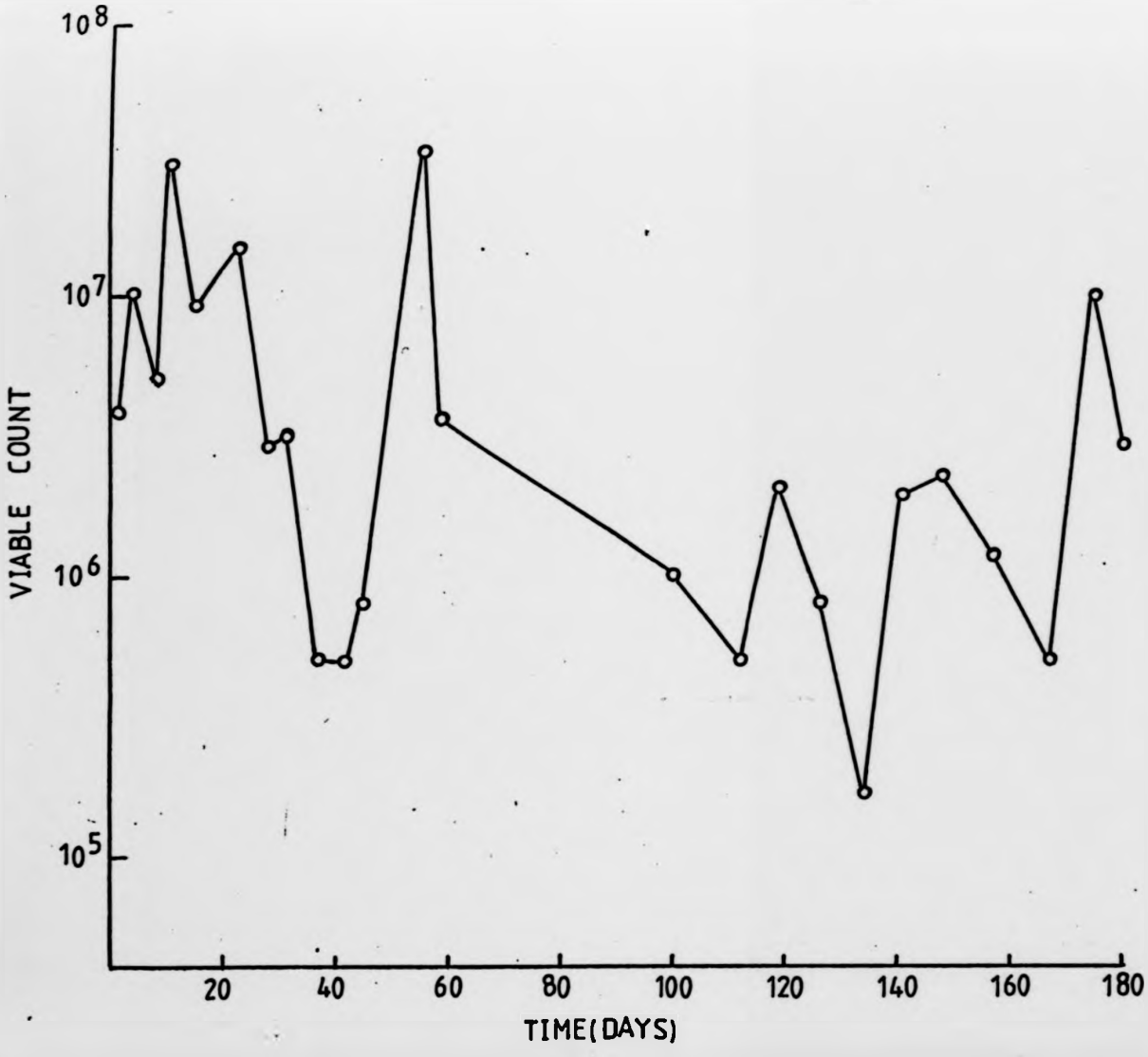

$10^8$
$10^7$
$10^6$
$10^5$
VIABLE COUNT
20
40
60
80
100
120
140
160
180
TIME(DAYS)

Figure 4.13 Viable count of organisms, growing on agar containing benzoate as the carbon source and 36DCPA as the nitrogen source, from the effluent of column B.

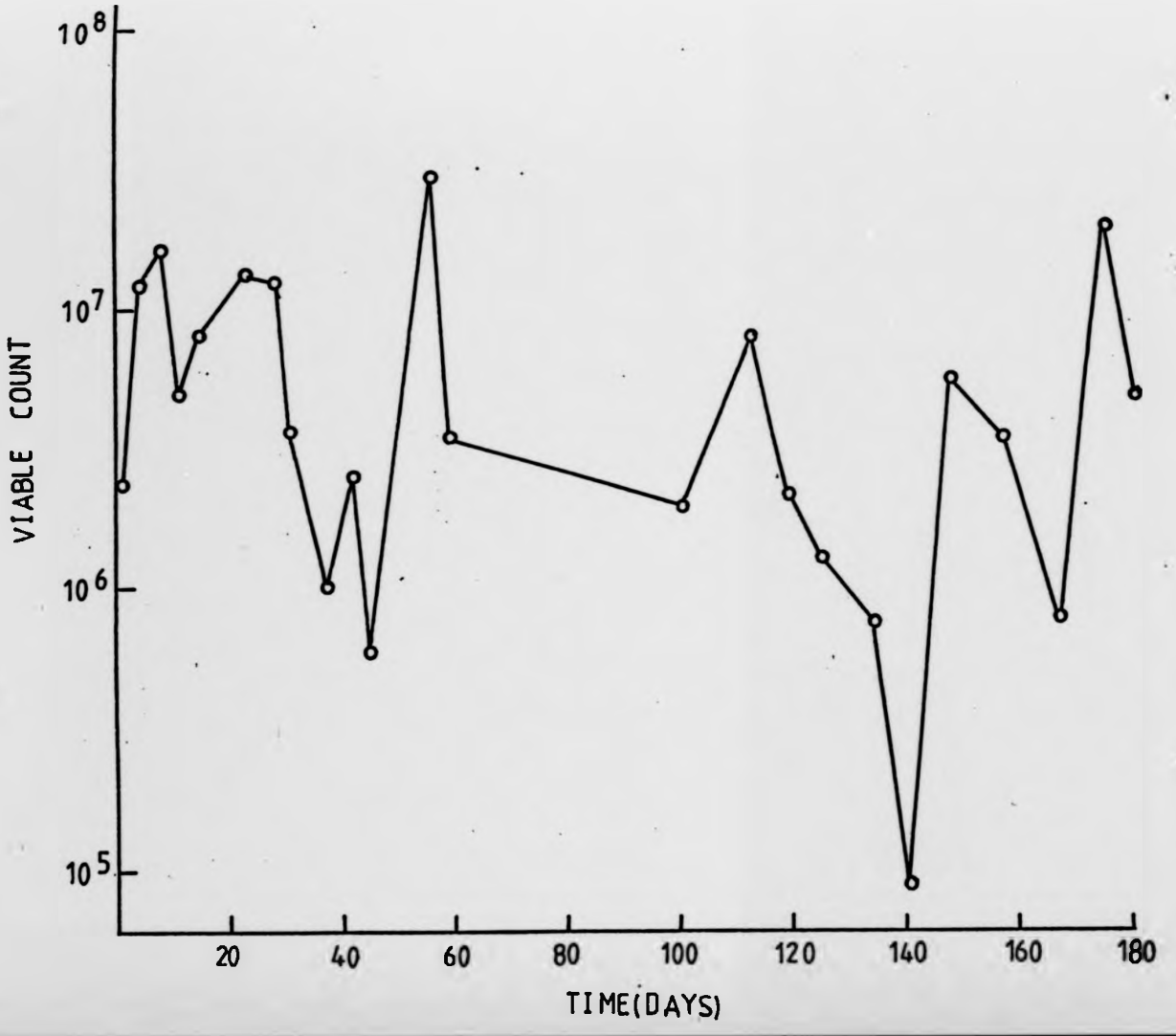
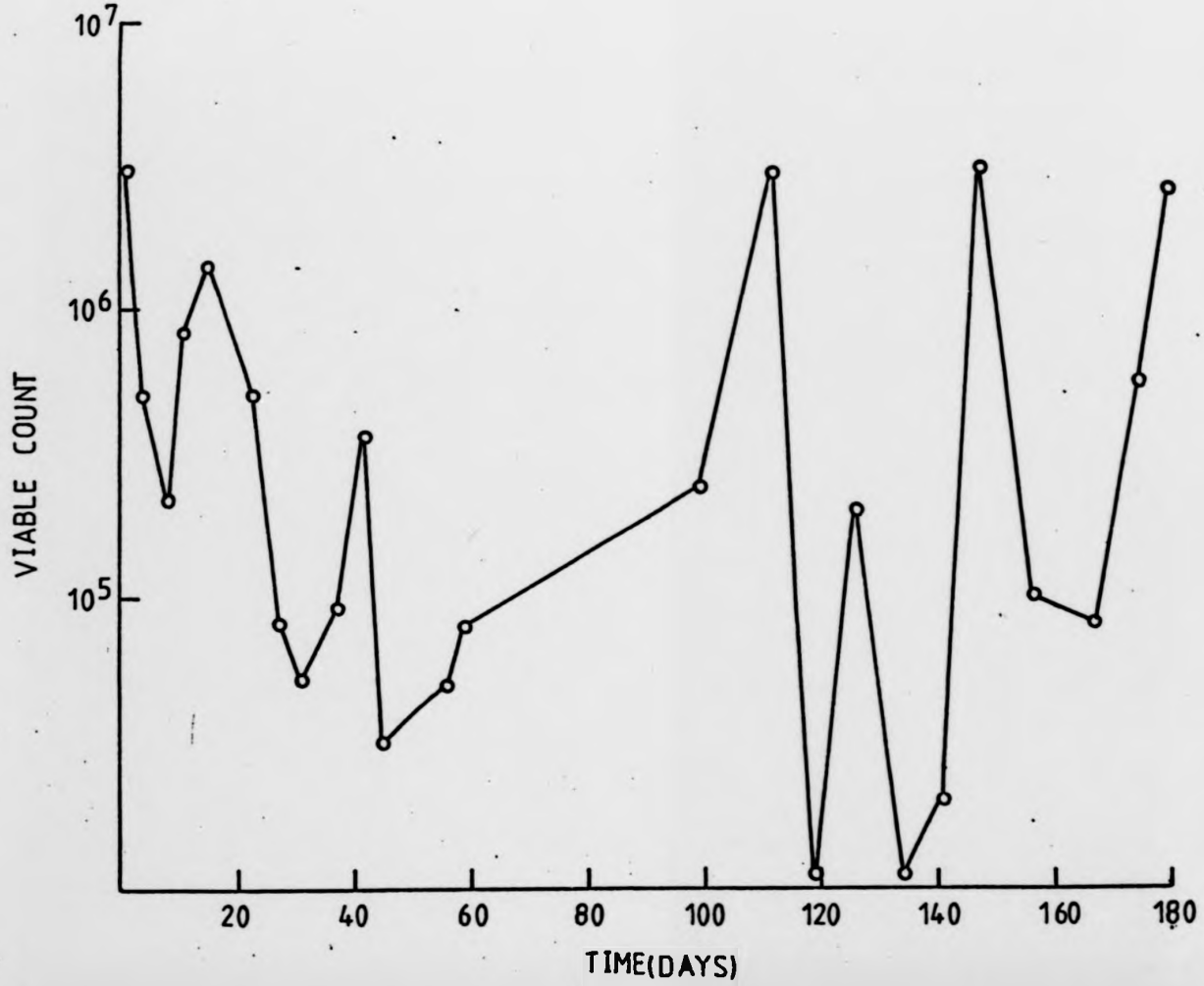

$10^8$
$10^7$
$10^6$
$10^5$
VIABLE COUNT
20
40
60
80
100
120
140
160
180
TIME(DAYS)

Figure 4.14 Viable count of organisms, growing on agar containing picolinate as the sole source of carbon and nitrogen, from the effluent of column B.


$10^7$
$10^6$
$10^5$
VIABLE COUNT
20
40
60
80
100
120
140
160
180
TIME(DAYS)

The inoculum consisted of effluent from columns A and B, and soil particles, also from columns A and B. A further quantity of soil (2g) that had not been treated with 36DCPA was also added to increase the variety of micro-organisms present initially, since some organisms would have been washed out from the soil columns by the 36DCPA treatement.

The organisms were allowed to grow up in closed culture, initially, before initiating the flow of influent medium to give a dilution rate of $0.032h^{-1}$.

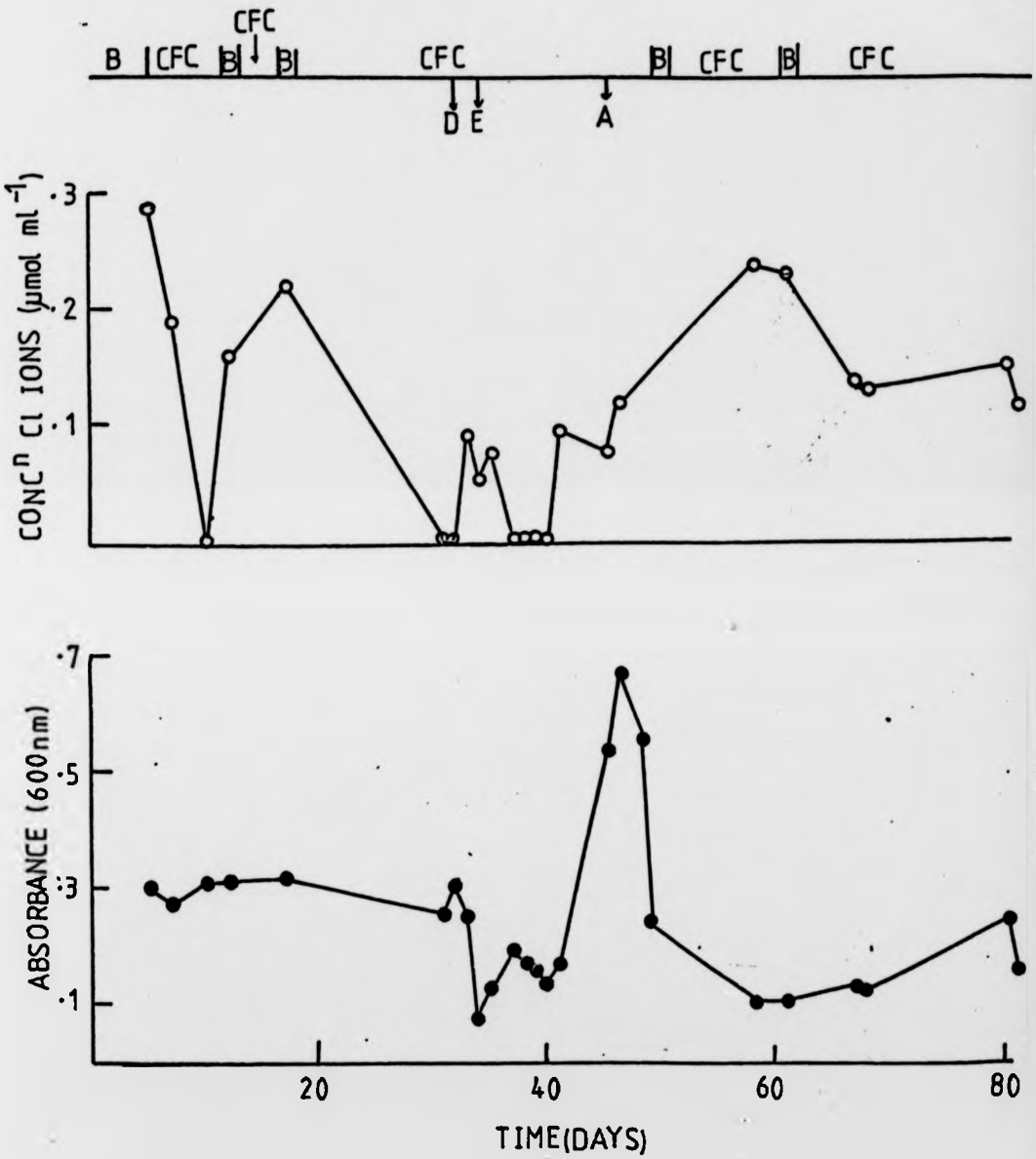
After 30 days growth the medium was changed to a much lower concentration of glycerol (0.1g carbon $l^{-1}$) whilst the 36DCPA concentration was increased to 0.5g carbon $l^{-1}$. These results are shown in Figure 4.15. All organisms isolated from the fermenter on agar containing glycerol and 36DCPA were not able, when inoculated into liquid culture containing 36DCPA alone, to liberate chloride ions from the 36DCPA present. It was inferred, therefore, that there was no breakdown of 36DCPA.

Benzoate was also used as a secondary source of carbon in this fermenter experiment. It was considered that the aromatic nature of the molecule would select for micro-organisms more likely to be able to attack 36DCPA.

The medium was changed on day 45 (figure 4.15) and a second portion of 2g soil was added to the fermenter to increase the variety of organisms present, as explained earlier.

This enrichment failed to select for any organisms capable of acting against 36DCPA or possessing any other desired capabilities.

Figure 4.15 Absorbance at 600nm (●) and free chloride ion concentration (○) for the chemostat enrichment experiements, D = .032 $h^{-1}$; CFC, growth in continuous flow; B, growth in batch; A, changed medium to benzoate and 36DCPA from glycerol and 36DCPA; D, medium changed from 0.1g carbon $l^{-1}$ 36DCPA and 0.5g carbon $l^{-1}$ glycerol to 0.5g carbon $l^{-1}$ 36DCPA and 0.1g carbon $l^{-1}$ glycerol; E, medium returned to 0.1g carbon $l^{-1}$ 36DCPA and 0.5g carbon $l^{-1}$ glycerol.


CFC
B
CFC
B
B
CFC
B
CFC
B
CFC
D
E
A
CONCⁿ Cl IONS (μmol ml⁻¹)
·3
·2
·1
ABSORBANCE (600nm)
·7
·5
·3
·1
20
40
60
80
TIME(DAYS)

## 4.2 DISCUSSION

Schlegel and Jannasch (1967) stated that "it is the aim and the art of enrichment culture technique to control those selection conditions which quickly and reproductively lead to the predominant population of one special organism, thereby facilitating its isolation". In the study performed the objective was to isolate not one single organism but one single metabolic capability, namely that of the degradation of 36DCPA.

If it is assumed that biodegradation is the ability of an experiment to find the right combination of organisms plus compound to be degraded plus environmental conditions (Kreuk and Hansveit, 1978) then by using a system which attempts, in all possible ways, to simulate the environment under which the compound has been seen to degrade (see section 3.1), the optimum conditions for biodegradation are likely to have been selected. It should be noted at this stage that Hungate (1961) has observed that it may be impossible in practice to make an identical replica of any particular ecological niche. In essence, all enrichment procedures follow the same general pattern. Any species which develops an advantage in some manner, whether it be a faster growth rate, a lower saturation constant for the substrate being studied, a higher tolerance to any toxic material present or produced, or any other advantage over its competitors, will establish a predominant population. In closed cultures the exhaustion of substrate and the build up of metabolic products will often result in a succession of predominant populations (eg see Horowitz, Gutnick & Rosenberg, 1975). In continuous culture, however, the turnover of substrate and the removal of metabolites and organisms, results in an elimination of the factors which lead to succession. Any beneficial metabolite produced, of course, will thus become limiting to the system.

Cassel, Sultzer & Lamb (1966) and Harder, Kuenen and Matin (1976) have reviewed microbial selection in continuous culture. Harder *et al* (1976) have concluded that "the ability to maintain, over long periods of time, a desired set of environmental conditions enables one to screen a large number of individual organisms with a view to selecting a mutant more adapted to those conditions". It might be suggested,

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that where an organism or a mutant is used, in the case of this study, a biochemical trait could be inserted, which in some cases cannot be ascribed to one organism (Munneke & Hsieh, 1974 and 1975). The experiments described in this chapter were all devised with the aim of producing a single biochemical trait, namely the degradation of 36DCPA, whether this be by a single organism of a mixed population. A complex degradation chain was expected since, it is known that the initial point of attack of organisms of unchlorinated picolinic acid is hydroxylation of the 6 position, and 36DCPA has a $Cl^-$ group at this position. The molecule can be considered relatively recalcitrant because of the 6 $Cl^-$ group. Chloride ion release might thus be a quick method of assessing the degradation of 36DCPA.

By comparing the release of chloride ions from the two columns it was possible to obtain an indication of the ability of the soil microflora to use 36DCPA as a nitrogen source. The average chloride ion concentration in the effluent medium from column A was 0.3 $\mu mol\ ml^{-1}$ and 0.15 $\mu mol\ ml^{-1}$ from column B. When these figures are calculated as a percentage of total possible chloride ion release, they are very comparable (1.9% and 1.6% respectively). The equivalence of these two parameters might suggest that at no time during the enrichment procedure was it easier for the microbial population to obtain their requirement for nitrogen from 36DCPA. It may be that the residual amount of available nitrogen in the soil was sufficient for the populations present. The low concentration of chloride ions released indicates that 36DCPA, under the conditions studied, exhibited a very slow decomposition rate. Figure 4.1 shows a marked relationship between chloride ions released and optical density at 600 nm. In batch experiments, using benzoate as a carbon source at $0.5gC\ l^{-1}$ it was discovered that the maximum absorbance obtainable was 0.6. Both columns had an average absorbance of 0.2, even though column A had a higher initial concentration of benzoate and could, theoretically, support a larger population. The relatively low absorbance in the column effluent would have been a reflection of many varied pressures on the populations such as predation or an inhibitory effect of 36DCPA. One problem with the column design which may have added to this effect was the formation of pellicles on the effluent tubing and on the sintered glass filter, which was placed on top of the soil to ensure even percolation of medium through the soil column.

The use of 36DCPA as a potential nitrogen source, or its lack of use, can be further examined by comparing the viable counts from both columns on the varying types of agar i.e by comparing figures 4.2 and 4.9, 4.3 and 4.10, 4.4 and 4.11, 4.5 and 4.12, 4.6 and 4.13 and 4.7 amd 4.14. It can be seen from these figures that there was a large fluctuation in the variable counts over the experimental period. There could be many reasons for this including straight competition for one limiting substrate or an effect of predation.

Another fact that is readily observable is the presence of organisms growing on 36DCPA agar with no other source of energy. Again the reasons for this could be many but the obvious answer to this problem is that suffficient material for the growth of the organisms was carried over onto the plate during the dilution process. It may also be that some of the organisms present were able to scavenge utilisable products from the hydrolysis of the agar itself.

Figures 4.7 and 4.14 show the growth of organisms on picolinate as the sole source of carbon and energy. It can be seen that the viable count is approximately 10 times lower than growth on other agars. This may indicate that the presence of a nitrogen atom in the aromatic nucleus confers some degree of recalcitrance on the molecule, since there was no such decimation during trial periods of growth on benzoate alone.

The populations growing on 36DCPA plus a supplementary carbon source may have been similar since it is possible that the presence of 36DCPA in the medium was inhibitory to some organisms.

It can be seen from Figure 4.15 that where the concentration of carrier substrate (glycerol) was lowered and 36DCPA was raised the absorbance immediately fell to a very low value. It was therefore considered that a selection process which was less demanding on the microorganism was needed. This is explained in Chapter 5.

One of the main problems with continuous enrichment for this type of study is inherent in the nature of that type of enrichment. Namely that the organisms in the soil are continually exposed to the herbicide. In the natural environment, however, the herbicide is annually applied. It is difficult, in this situation therefore, to extrapolate the results exhibited here to the ecological niche of the field since 36DCPA is degraded slowly (see section 3.1) it may be that the entire application will be mineralised in one year.

In these experiments a very high level of 36DCPA, expressed as gC $l^{-1}$, was used. It may have been to some advantage to undertake a study where low levels of carbon were available to the organisms but this type of enrichment is difficult since there is a difficulty in monitoring biomass at low levels.

CHAPTER 5

# THE ISOLATION OF A MICROBIAL COMMUNITY ABLE TO GROW ON PICOLINATE, ITS IDENTIFICATION, AND ITS ADAPTATION TO TOLERATE 3,6-DICHLOROPICOLINATE

## 5.1 RESULTS

Since direct enrichments for microorganisms, capable of utilising 36DCPA were unsuccessful (Chapter 4) the method of analogue enrichment (Focht & Alexander, 1970) was used.

Any organism, or group of organisms, that were able to utilise unchlorinated picolinate might have a selective advantage over other organisms when exposed to 36DCPA. It might be possible for an organism to evolve a novel enzyme capable of dechlorinating 36DCPA similar to that described by Senior, Bull & Slater (1976).

### 5.1.1 Isolation and Adaptation of the Microbial Community

The apparatus used was an LH Engineering series 500 continuous flow system described in section 2.3.2. The organisms isolated were grown initially on the defined medium described in section 2.1.1. supplemented with picolinate as the sole source of carbon and energy at a concentration of 0.5g carbon $l^{-1}$. The dilution rate, and hence growth rate, used for the enrichment was $0.08h^{-1}$.

2g of soil from column A (see section 4.1.1.1.) and 2g of soil sampled from Warwick University campus (to represent soil with a full complement of microorganisms) were used for the inoculum. It was placed into the culture vessel containing 800ml of defined medium with picolinate as the sole carbon source. The organisms in the soil were grown up in closed culture initially until the culture was visibly turbid. Medium flow was initiated at a rate of approximately 68ml $h^{-1}$. The parameters measured were absorbance at 600nm, viable count on nutrient agar and viable organisms capable of growing on picolinate agar, with and without another source of nitrogen. The culture vessel was sampled on a regular basis every two or three days.

At certain stages in the growth of the population, the organisms were exposed to 36DCPA in the medium at the same time as picolinate, namely at days number 15, 62, 130. The concentration of 36DCPA used was 0.1g carbon $l^{-1}$. The concentration of picolinate remained unchanged.

Figure 5.1 shows the changes in absorbance of the population over the time period studied. The regions marked P show time intervals where the organisms were growing on picolinate alone. The regions marked P + 36DCPA were time regions where the organisms were growing on picolinate and 36DCPA combined.

The organisms were allowed to grow for 15 days on picolinate alone to allow any organisms that were only loosely attached to the picolinate utilisers to wash out. The medium was changed to the mixture of carbon sources. The absorbance dropped rapidly from 0.65 to 0.27 within 150h at a steady rate. The medium was changed back to picolinate alone before loss of any members of the community could occur.

After 62 days the medium composition was again changed to the mixed substrates. The absorbance dropped significantly again, from 0.64 to 0.215. The rate of decrease was, however, much slower this time taking 31 days.

After a third period of growth on picolinate alone, the medium was changed to picolinate plus 36DCPA. The absorbance of the culture fluctuated extensively from day to day but after a period of approximately 50 days the fluctuations became less pronounced and the absorbance became steady at approximately 0.45.

Figure 5.2 shows the viable count of organisms from the culture vessel on nutrient agar. This figure also shows the marked decrease of organisms after switching from medium containing picolinate alone to medium containing picolinate and 36DCPA at days 15, 62 and 130. After the final change to the mixed medium, the populations oscillated markedly but the amplitude of the oscillations did decrease with time, indicating a stabilisation of the changes occurring within the population. The viable count $ml^{-1}$ levelled out to approximately $1.1 \times 10^8$ organisms $ml^{-1}$.

Figure 5.1 Absorbance of 600nm of the mixed population of microorganisms. The sections labelled P show the time intervals when the population was growing on picolinate alone. The sections labelled P + 36DCPA show the intervals where the population was growing on picolinate +36DCPA.

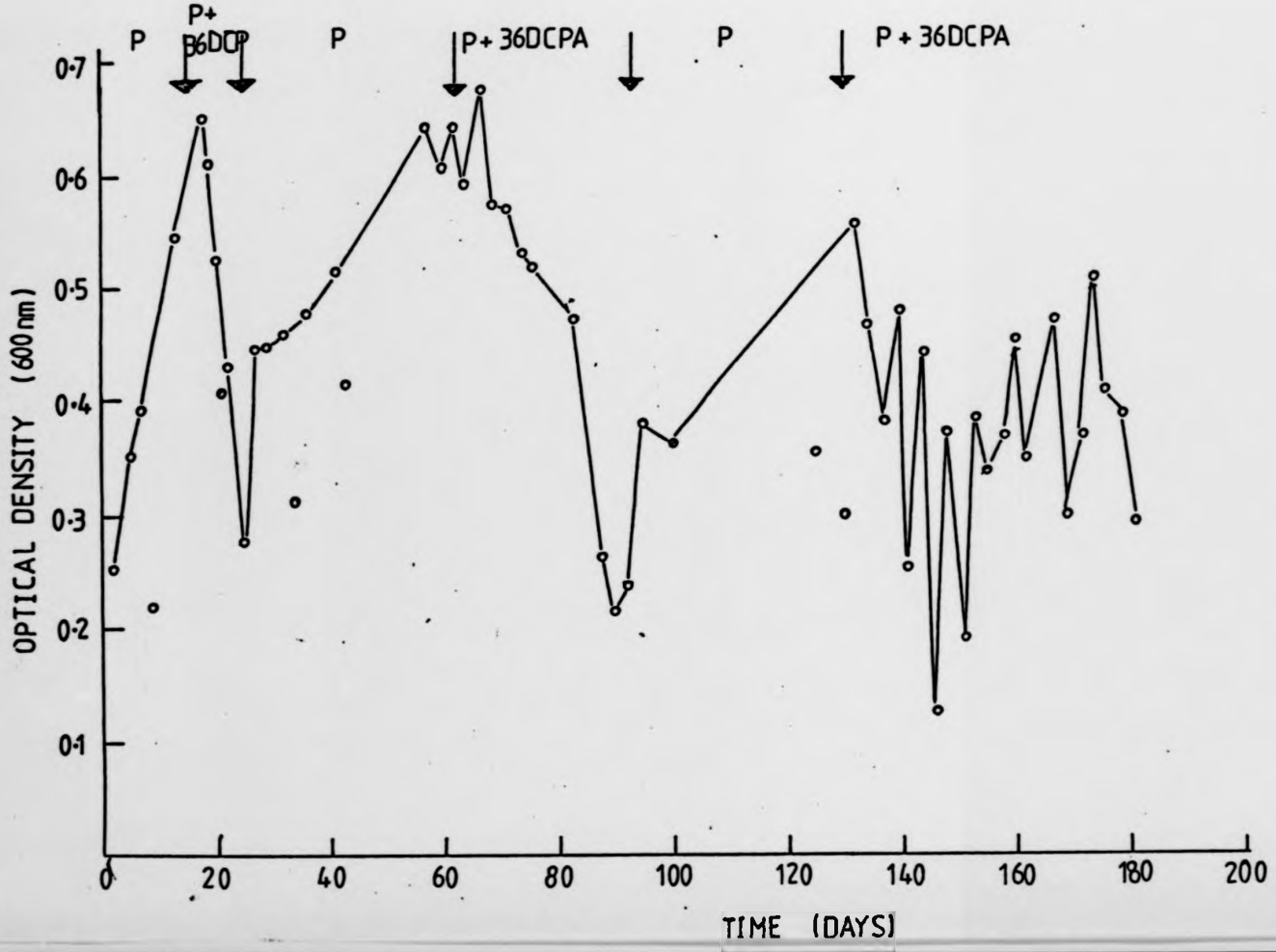
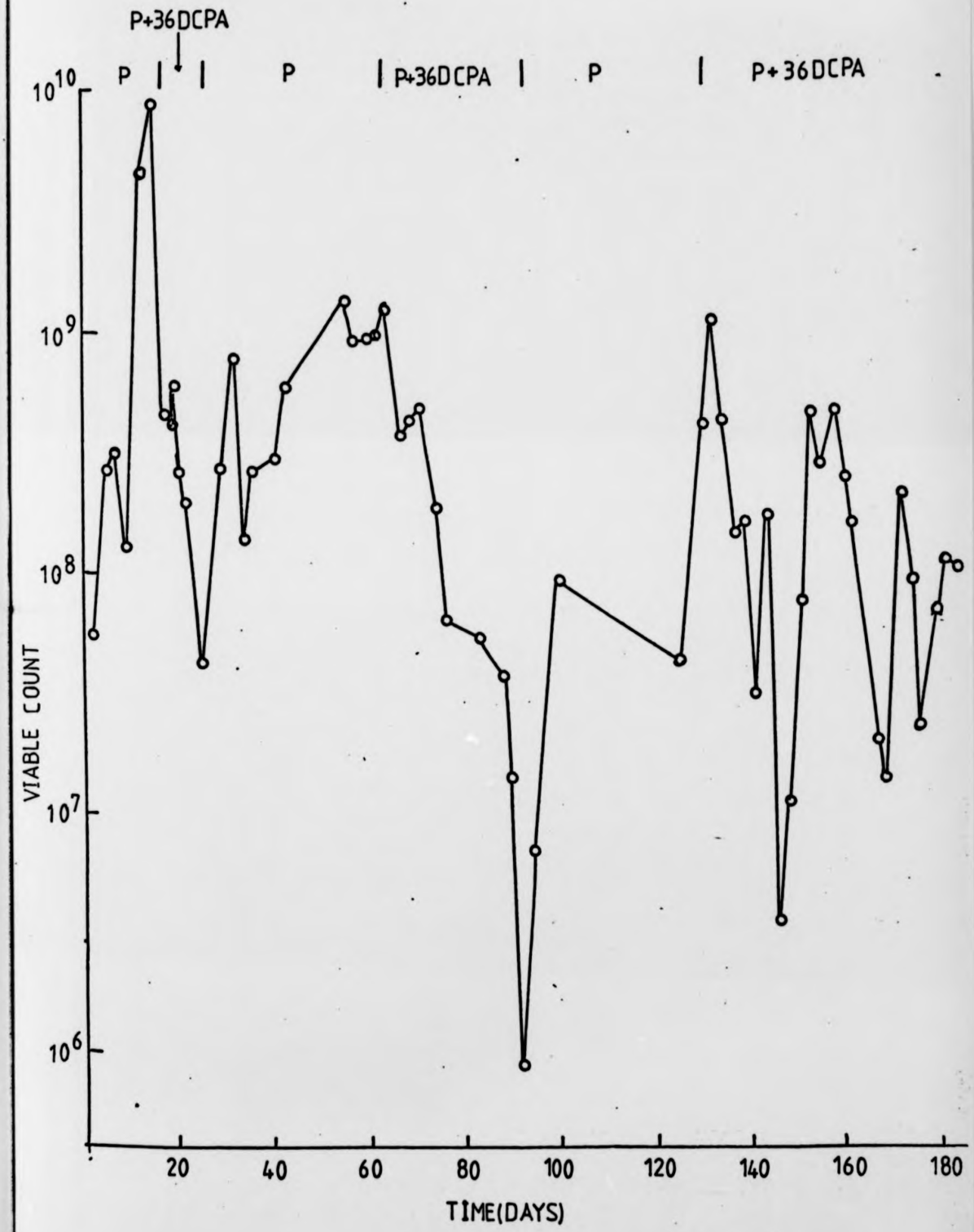

P
P+ 36DCP
P
P+ 36DCPA
P
P + 36DCPA
OPTICAL DENSITY (600nm)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
20
40
60
80
100
120
140
160
180
200
TIME (DAYS)


Figure 5.2 Changes in viable count of organisms on nutrient agar whilst growing on picolinate as the source of carbon and energy.

P indicates the time intervals when the population was growing on picolinate alone. P + 36DCPA indicates the time intervals when the population was growing on picolinate + 36DCPA.


P+36DCPA
P
P
P+36DCPA
P
P+36DCPA
$10^{10}$
$10^{9}$
$10^{8}$
$10^{7}$
$10^{6}$
VIABLE COUNT
20
40
60
80
100
120
140
160
180
TIME(DAYS)

Figures 5.3 and 5.4 show the viable count of picolinate utilises over the same adaptation period. Figure 5.3 shows growth on picolinate plus ammonium ions and figure 5.4 shows growth on picolinate without ammonium ions. Both figures show the marked depression of viable numbers of organisms in the period when they were growing on mixed substrates. The graphs also show the damping of the oscillations in viable count after the culture had adapted to grow with 36DCPA in the influent medium.

There were six organisms isolated from the mixed culture. There were designated A, B, D, E, G and R and all six organisms were present in the mixed culture before and after adaption to 36DCPA. Three of these organisms A, B and D were isolated on agar containing picolinate and so were termed primary utilisers, that is, organisms that could use picolinate as the sole source of carbon and energy. It was later found that organism A was not able to grow in liquid culture containing picolinate nor subcultured onto picolinate plates. This would seem to indicate that the organism could not utilise picolinate but was growing on the agar by scavenging materials from the agar gel, or from the other primary utilisers, or from carryover products.

Organism A was also inoculated into liquid medium supplemented with 6 hydroxy-picolinate (see section 2.7.2) and picolinate plus yeast extract (0.1% w/v) to determine whether it required a co factor, vitamin or if the cell was somehow impermeable to picolinate itself. However in both these cases there was no growth.

Organism D was also very difficult to grow on picolinate in liquid culture due to its very slow growth rate (colony size 0.5mm in 4-5 days).

The other three organisms E, G and R could not grow on picolinate and were termed secondary utilisers. Their growth was on breakdown or lysis products of the primary utilisers.

Table 5.1 shows the percentage of different organisms that grew on nutrient agar plates over the adaptation period. Where 0% is given this indicates that none of that particular organism grew on the lowest dilution that was plated out. It does not indicate that these organisms were completely absent from the growing mixture.


Figure 5.3 Growth of the mixed population of microorganisms on agar containing picolinate, as the sole source of carbon, and ammonium ions. The section labelled P indicates growth on picolinate alone. The section labelled P + 36DCPA indicates growth on picolinate + 36 DCPA.

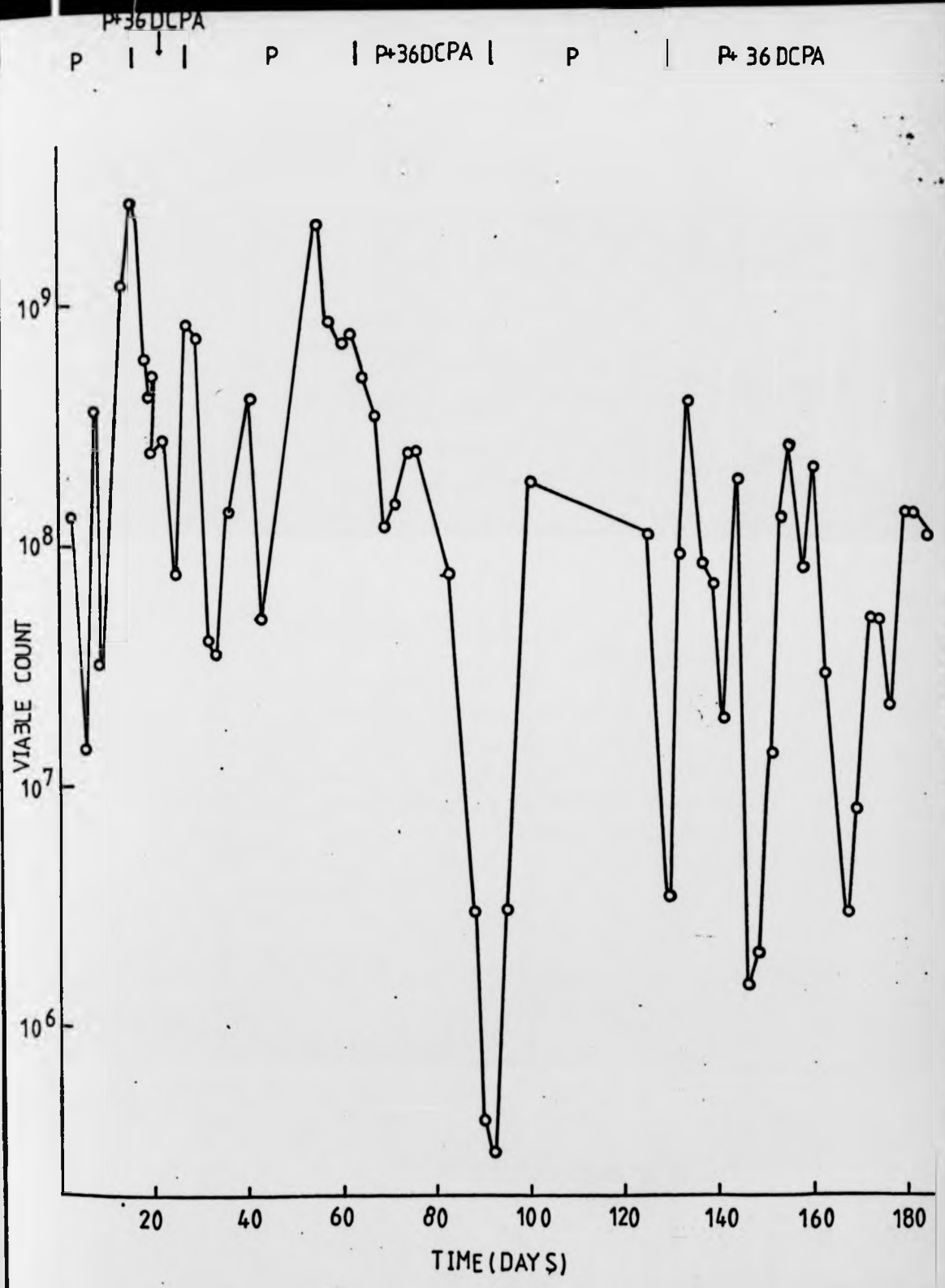

P+36DCPA
P
P
P+36DCPA
P
P+36DCPA
$10^9$
$10^8$
$10^7$
$10^6$
VIABLE COUNT
20
40
60
80
100
120
140
160
180
TIME (DAYS)

Figure 5.4 Growth of the mixed population of microorganisms on agar containing picolinate as the sole source of carbon and nitrogen. The section labelled P indicates growth on picolinate alone. The section labelled P + 36DCPA indicates growth on picolinate + 36DCPA.

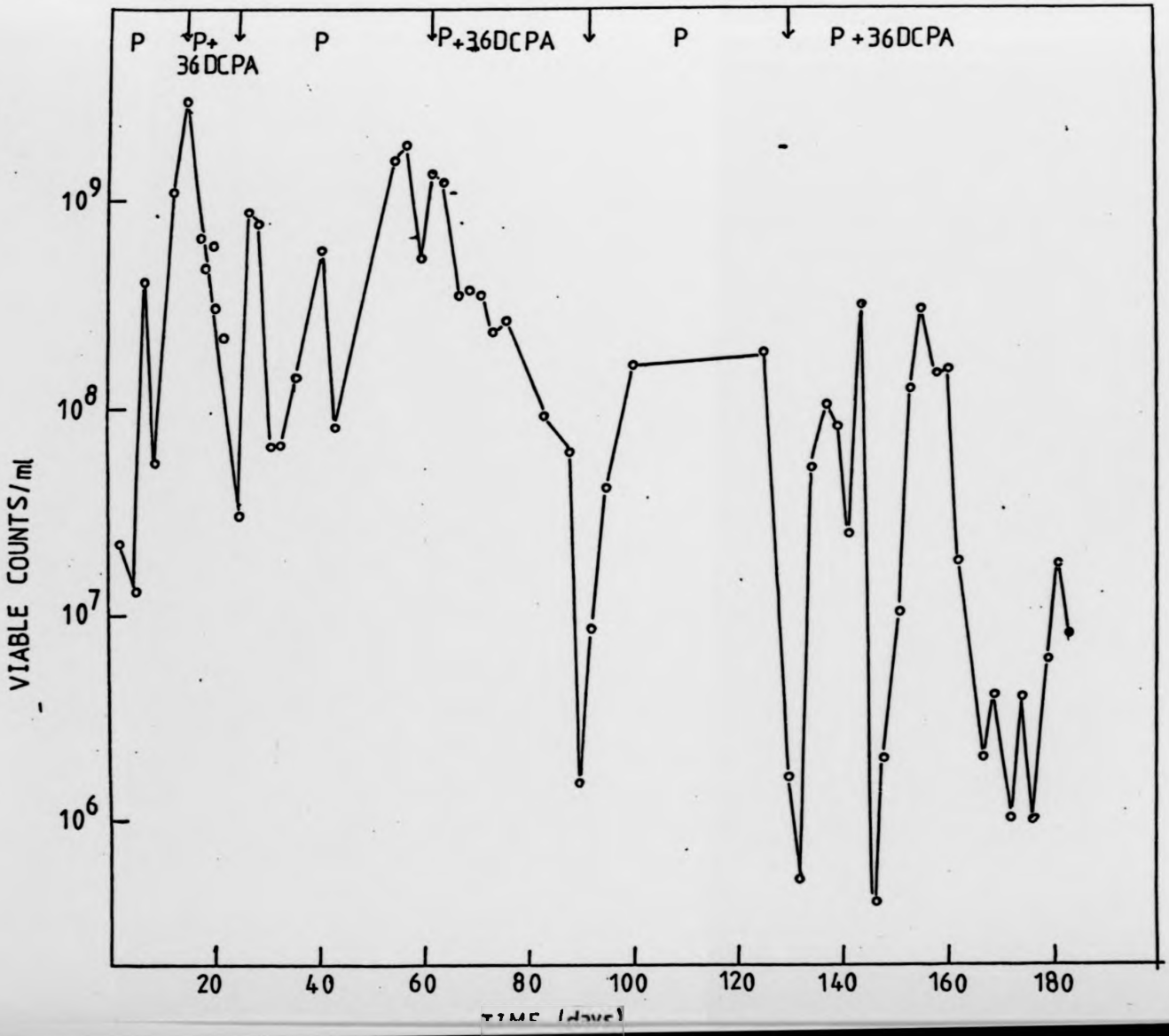

P
P+ 36DCPA
P
P+36DCPA
P
P+36DCPA
VIABLE COUNTS/ml
$10^9$
$10^8$
$10^7$
$10^6$
20
40
60
80
100
120
140
160
180
TIME (days)

TABLE 5.1

The percentage of individual organisms in the mixed culture during its adaptation to tolerate 36DCPA. (Identification was made on colony morphology alone).

| | ORGANISMS | | | | | |
|---|---|---|---|---|---|---|
| DAY | A | B | D | E | G | R |
| 32 | 1.25 | 8.75 | 66.25 | 18.75 | 5 | 0 |
| 34 | 2 | 39 | 37 | 0 | 19 | 3 |
| 36 | 4 | 41 | 46 | 4 | 4 | 1 |
| 41 | 0 | 70 | 25 | 0 | 4 | 1 |
| 43 | 0 | 16 | 76 | 0 | 8 | 0 |
| 55 | 18 | 73 | 0 | 0 | 9 | 0 |
| 57 | 0 | 60 | 20 | 0 | 20 | 0 |
| 60 | 0 | 67 | 0 | 11 | 22 | 0 |
| 62 | 4 | 96 | 0 | 0 | 0 | 0 |
| 64 | 6 | 94 | 0 | 0 | 0 | 0 |
| 67 | 25 | 67 | 8 | 0 | 9 | 0 |
| 69 | 8 | 79 | 0 | 10 | 3 | 0 |
| 71 | 0 | 98 | 0 | 0 | 2 | 0 |
| 74 | 0 | 100 | 0 | 0 | 0 | 0 |
| 76 | 2 | 98 | 0 | 0 | 0 | 0 |
| 83 | 0 | 92 | 0 | 6 | 2 | 0 |
| 90 | 1 | 98 | 0 | 0 | 1 | 0 |
| 95 | 25 | 50 | 0 | 25 | 0 | 0 |
| 100 | 9 | 34 | 0 | 47 | 9 | 0 |
| 125 | 2 | 75 | 0 | 14 | 9 | 0 |
| 132 | 0 | 13 | 84 | 0 | 4 | 0 |

Table 5.1 cont'd

| | ORGANISMS | | | | | |
|---|---|---|---|---|---|---|
| DAY | A | B | D | E | G | R |
| 134 | 0 | 0 | 95 | 0 | 5 | 0 |
| 137 | 0 | 78 | 3 | 0 | 19 | 0 |
| 139 | 0 | 43 | 33 | 19 | 5 | 0 |
| 144 | 0 | 65 | 0 | 0 | 33 | 2 |
| 146 | 9 | 26 | 58 | 0 | 4 | 3 |
| 148 | 12 | 27 | 22 | 24 | 12 | 3 |
| 151 | 2 | 85 | 0 | 0 | 13 | 0 |
| 153 | 2 | 23 | 27 | 0 | 49 | 0 |
| 155 | 0 | 92 | 0 | 0 | 9 | 0 |
| 158 | 0 | 39 | 54 | 0 | 7 | 0 |
| 160 | 0 | 73 | 5 | 0 | 22 | 0 |
| 162 | 0 | 55 | 0 | 0 | 40 | 5 |

* Where 0 is given as the percentage there were none of these organisms on the lowest dilution of plates, i.e. is equivalent to less than 0.1%.

It can be seen from table 5.1 that organism B was the predominant member of the mixed culture for most of the adaption period. This reflected the dependance of the other organisms on the actions of organism B.

During the first period of growth on the mixed substrates (Day 62-Day 92) the percentage of organisms D and G dropped significantly and did not recover until the medium had been changed back to picolinate alone. However, during the final period of growth on the mixed substrates the percentage of these two organisms increased. This may indicate that the mechanism for tolerating 36DCPA was connected, in some way, to these two organisms.

The distribution of levels of organism E was similar to those of organisms D and G but towards the end of the study period (Day 151) the percentage of this organism dropped to zero.

Organism R appeared at very low levels throughout the entire experiment, as did organism A.

The average percentage of the organisms in the mixed population is given in table 5.2.

Although it was possible to achieve a steady state in terms of dry weight and absorbance there were, however, significant fluctuations in the species composition (see Chapter 6).

### 5.1.2 Identification of the Organisms in the Mixed Culture

All isolates were subjected to morphological, physiological and biochemical tests in order to identify them. This work was carried out in collaboration with the Torrey Research Station (National Collection of Industrial Bacteria), Aberdeen.

TABLE 5.2

Average percentage of organisms in the mixed population

| Organism | Av. %age |
|---|---|
| A | 4 |
| B | 59.8 |
| D | 20.0 |
| E | 5.4 |
| G | 10.2 |
| R | 0.5 |

5.1.2.1 Morphological Tests

5.1.2.1.1 Organisms A

The colonies of organism A were greenish-blue in colour, flat, rough, circular and translucent. They grew to a size of 1mm in 1 day. The organisms were Gram-negative, fairly short rods with flagella. Spores were not observed. The colonies were easily identifiable on nutrient agar since they extended over the agar much more extensively than the other organisms.

5.1.2.1.2 Organism B

The colonies of organism B were the only large colonies that grew on picolinate (Organisms A and D had very small colonies on picolinate agar). They were off-white in colour, smooth, circular low convex and translucent. They grew to a size of 0.5mm in 1 day. The organisms were Gram-negative, short rods and coccobacilli. They exhibited peritrichous flagella. Spores were not observed.

5.1.2.1.3 Organism D

The colonies of organism D were easily identifiable due to their small size. They grew to 0.5mm diameter in 4-5 days. The colonies were off-white in colour, entire, flat, smooth, circular and translucent. The organisms were Gram-negative short rods with some angular pairs. The organism had peritrichous flagella. Spores were not observed.

5.1.2.1.4 Organism E

Colonies of organism E were off-white in colour, low convex, rough, circular and opaque. They grew embedded into the agar surface producing 1mm diameter sized colonies in 1 day. The organisms were Gram-negative rods and were flagellate. The spores observed were central and unswollen.

5.1.2.1.5 Organism G

The colonies of organism G were pale yellowish in colour, entire, flat, smooth, circular and translucent. They grew to a size of 0.75mm in 1 day. The organisms were Gram-negative short rods with peritrichous flagella and without spores.

5.1.2.1.6 Organism R

The colonies of organism R were orange in colour, entire, convex, rough, circular and almost opaque. They grew to 2mm diameter in 1 day. The organisms were Gram-positive rods without flagella or spores.

5.1.2.2 Physiological Tests

The growth of the six organisms was measured at varying temperatures. Table 5.3 summarises the data obtained. The organisms were normally grown at 30°C.

5.1.2.3 Biochemical Tests

From the morphological, physiological and preliminary biochemical tests (oxidase, catalase and oxidation/fermentation of glucose, see Table 5.4) the organisms were split into three groups. A, B and D were Gram-negative non-fermenters, E was a Bacillus and G and R were Corynebacteria. These three groups were examined separately for a number of the biochemical tests. Table 5.4 shows the tests used to identify the Gram-negative non-fermenters. Tests were carried out as indicated in Cowan & Steel (1974). From this data it was concluded that organism A was Pseudomonas aeruginosa, organism B was Alcaligenes faecalis and organism D was a second Alcaligenes sp.

Table 5.5 shows the biochemical tests carried out on the Bacillus sp and from the results it was concluded that organism E was B.licheniformis.

TABLE 5.3

Growth of the six bacteria at elevated and lowered temperatures

ND indicates a test was not performed.

1 Trace growth after 1 month

2 In 1 month

+/- Indicates trace growth

| ORGANICS | TEMPERATURE °C | | | | | | |
|---|---|---|---|---|---|---|---|
| | 5 | 30 | 37 | 42 | 45 | 50 | 60 |
| A | + | + | ND | + | + | - | ND |
| B | + | + | + | +/- | - | ND | ND |
| D | +/-[1] | + | +/- | - | ND | ND | ND |
| E | ND | + | ND | ND | + | + | - |
| G | +[2] | + | + | - | - | ND | ND |
| R | ND | + | +/- | - | ND | ND | ND |

TABLE 5.4

Biochemical Tests on gram negative non fermentative organisms A, B, D

| Test | A | B | D |
|---|---|---|---|
| Catalase | + | + | + |
| Oxidase | + | + | + |
| O/F gluc | O | - | - |
| Pyocyanin | + | - | - |
| Fluorescence | + | - | - |
| D.L. Arg. C.S.U | + | - | - |
| Betaine C.S.U | + | - | - |
| Glucose C.S.U | + | - | - |
| Lactate C.S.U | + | + | - |
| Acetate C.S.U | + | + | - |
| Penicillin G | - | - | - |
| Streptomycin | + | - | + |
| Chloramphenicol | + | + | + |
| Tetracycline | + | + | + |
| Novobiocin | - | - | - |
| Polymyxin B | + | + | + |
| Levan | - | - | - |
| Acid from Glucose | + | - | - |
| Gas from Glucose | - | - | - |
| ONPG | - | - | trace |
| Arginine/Moller | + | - | - |
| Lysine/Moller | - | - | - |

TABLE 5.4 cont'd

| Test | A | B | D |
|---|---|---|---|
| Ornithine/Moller | - | - | - |
| $NO_3$ to $NO_2$ | - | + | - |
| $NO_3$ to $N_2$ | + | - | - |
| DNA ase | + | - | - |
| Casein | + | - | - |
| Starch | - | - | - |
| Lecithin egg | - | - | - |
| Lipase egg | + | - | - |
| $NH_3$ | + | - | - |
| Indole | - | - | - |
| MR | - | - | - |
| VP | - | - | - |
| 3 Ketolactose | NP | - | - |

TABLE 5.5

Biochemical Tests carried out on Organism E

| Test | Result |
|---|---|
| Spore shape | elliptical |
| Sporangium distented | - |
| Spore position | central |
| Catalase | + |
| Anaerobic Growth | + |
| Oxidase | + slow |
| Growth in 5% NaCl | + |
| " 7% NaCl | + |
| " 10% NaCl | + |
| " pH 5.7 Broth | + |
| Acid from Glucose | + |
| Gas from Glucose | - |
| V.P. (acetoin) | + |
| Egg yolk agar opacity | - |
| Casein decomposition | + |
| Gelatin decomposition | + |
| Starch hydrolsis | + |
| $NO_3$ to $NO_2$ | + |
| Indole | - |
| Kosers Citrate | + |
| Arginine dihydrolase/ Mollers | + |
| pH in V.P. Broth | > 8.0 |

Table 5.6 shows the biochemical tests carried out on the Corynebacteria. From the results organism R was concluded to be a Rhodococcus sp. The classification of organism G was rather difficult. Opinion is growing that coryneform bacteria of different cell wall composition should be in different genera. At present, however, there is no genera for DAB - containing coryneforms. Most of these organisms are at present included in the genus Corynebacterium (Keddie & Cure, 1977). Organism G was, therefore, designated to be a Corynebacterium sp.

## 5.2 DISCUSSION

Frances, Spanggord, Ouchi and Bohonos (1978) have stated that "cometabolism appears to occur widely in nature". They proceeded to describe a Pseudomonas sp. which metabolised p,p'-dichlorophenyl analogues of DDT only in the presence of diphenylethane. The inference was that the diphenylethane induces enzymes for its own utilisation and these enzymes were unable to distinguish between the raw substrate and its chlorinated analogue resulting in the production of some chlorinated metabolites indicating degradation of the halogenated molecule.

This phenomenon, however, was described by Jensen (1963) who found that a strain of Pseudomonas dehalogens which utilised oxalate, released chloride ions from trichloroacetate.

In the experiments that are described in this chapter, an experimentally stable community was exposed to a chlorinated analogue of the main carbon source to produce a second community which was capable of dehalogenation. The community isolated, which could metabolise picolinate as the sole source of carbon, was relatively stable. In the chemostat, the population reached what may be termed a pseudo steady state, i.e. the growth rate of the whole population was equal to the dilution rate of the vessel contents. The culture can be described as a pseudo-steady state since although the whole population appeared to reach a steady state, its component populations showed marked degrees of change. These dynamic fluctuations may dictate that

TABLE 5.6

Biochemical Tests on the Coryneform Isolates

| Test | G | R |
|---|---|---|
| D.L. Arginine C.S.U | + | NT |
| Glucose C.S.U. | + | + |
| Lactate C.S.U. | NT | + |
| Penicillin G | + | NT |
| Streptomycin | - | NT |
| Chloramphenicol | + | NT |
| Tetracycline | - | NT |
| Novobiocin | - | NT |
| Polymyxin | + | NT |
| Levan | - | NT |
| Oxidase | - | - |
| Catalase | + | + |
| Acid from glucose | + | - |
| Gas from glucose | - | - |
| ONPG | + | NT |
| Arginine/Moller | - | NT |
| Lysine/Moller | - | NT |
| Ornithine/Moller | - | NT |
| $NO_3$ to $NO_2$ | - | - |
| DNA-ase | + | - |
| $NO_3$ to $N_2$ | - | - |
| Casein | - | - |
| Starch | - | - |
| Lecithin egg | - | - |
| Lipase egg | - | - |

TABLE 5.6 cont'd

| Test | G | R |
|---|---|---|
| $NH_3$ | - | NT |
| Indole | - | NT |
| MR | - | NT |
| VP | - | NT |
| NaCl 10% growth | NT | + |
| Urease | NT | + |
| Anaerobic growth | NT | - |
| Cell wall diamino acid | D.A.B | Meso D.A.P |
| Cell wall sugar | galactose | NT |
| Fatty acid profile | of Coryne-bacterium aquaticum type | typical of Rhodococcus |

the system never reaches a true steady state. Indeed, Cassel Sultzer & Lamb (1966) have concluded that "At various constant detention times (in a chemostat) all parameters which reflected biological activity fluctuated continuously. Thus continuous mixed cultures must be considered as dynamic systems." So, in terms of these experiments, a steady state must be defined as a period of growth during which time the total biomass production was equal to the substrate input to the fermenter. Kroess (1977) has stated that "small variations in species composition will mostly leave the ecosystem intact as a whole", which further supports this definition of a steady state.

The rapid decrease in absorbance after the first two changes of media, i.e. at times when 36DCPA was present in the medium, showed that the population was inhibited, in some way, by the 36DCPA since the level of picolinate was not altered. If the herbicide has no adverse effects on the population then it might be assumed that the absorbance would have remained stable since the system was in a steady state (as defined above). The mechanism of this inhibition may be explained as follows. It is known that the first step in the breakdown of picolinate is hydroxylation of the 6 position to produce 6-hydroxy-picolinate. It is possible that the hydroxylase which performed this transition was not capable of distinguishing between picolinate and 36DCPA and so bound both types of molecule. The hydroxylase may then not have been able to release the 36DCPA since without dechlorinisation, hydroxylation could not continue, due to the totally different nature of the chloride ion and the hydrogen ion.

After the third period of media changes, however, it is suggested that the hydroxylase may have evolved slightly in order that release of the 36DCPA was possible, or that release of the chloride ion to a lesser degree was also possible.

The mechanism described was attributed more the second Alcaligenes sp. and the Corynebacterium since the percentage of these organisms in the mixed culture increased after the adaptation period had passed.

However, the percentage of A. faecalis was still relatively high. Since there were two primary utilisers, notably Alcaligenes sp2. and A. faecalis, and it is considered that complete competitors cannot coexist, then there must have been some form of interaction between the two species. It would be interesting to study the nature of this interaction.

This facet of the microbial community having more than one primary population able to grow alone on the growth limiting substrate is a common feature of many microbial communities isolated, and the interactions between these populations tends to stabilise the systems. Examples of communities with more than one primary utiliser have been shown by Senior, Bull & Slater (1976), Sinerez & Pirt (1977) and Parkes, Minney, Bull & Slater (personal communication).

The population was monitored on agar plates without ammonium ions in order to try and promote ring cleavage since the only N atoms present were those in the ring structure. Houghton & Cain (1972) found a Nocardia species that was capable of utilising pyridine as sole carbon, nitrogen and energy source.

Pseudomonas sp. have been found to be capable of degrading many xenobiotic compounds in nature. Knackmuss & Hellwig (1978) have found a Pseudomonas sp. capable of utilising chlorinated phenols, and Mallory & Matsumura (1967) found a variety of P.melophthora, the bacterial symbiont of the apple maggot (Rhagoletis pomonella), had demonstrable activities against six insecticides including parathion, dieldrin and diazinon. 3,4-dichloroaniline (Suroutseva & Funtikova (1978), malathion, (Matsurmura & Boush 1966), dalapon (Senior et al 1976), phthlate esters (Kurane, Suzuki and Takahara 1977), benzyl penicillin (Johnsen 1977) and DDT analogues (Francis et al 1978), have all been described as substrates for various identified and unidentified species of Pseudomonas. It is not surprising, therefore, to find a Pseudomonas sp. in this case P.aeruginosa, present in the microbial community capable of degrading 36DCPA.

Alcaligenes sp. have also been reported to degrade a variety of xenobiolic compounds. Dorn & Knackmuss (1978) have described a species of Alcaligenes which was capable of degrading fluoro- and bromo-catechols by a pyrochatechase produced in benzoate grown organisms. Furukawa, Matsumura & Tanomura (1978) have described an Alcaligenes sp. which was capable of cleaving one ring in the compound 4-chlorobiphenyl. Furukawa, Tanomura and Kamibayashi (1978) have used the same strain to study the degradation of 31 different polychlorinated biphenyls, although the rate of degradation was much reduced where chlorine substitutions were greater than 4.

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One of the many problems associated with this type of adaption experiment can be observed by a cursory glance at Table 5.1. This is that some of the organisms, most noticably organism R, Rhodococcus sp. were not detected on every sample of the fermenter. This was due to the organisms being relatively scarce in the mixed population and even at the lowest serial dilution there were no organisms in the plated out media. A further problem highlighted by the absence of some organisms on serial dilution is that of fastidious organisms and one question that must be asked is "were the selection techniques broad enough to detect any organism that might have been present". Since growth was measured on nutrient agar as well as many other carbon sources it was felt that the whole variety of organisms present in the community were isolated and that the six membered community isolated was an actual representation of the fermenter contents as a whole.

CHAPTER 6

# CHEMOSTAT STUDIES WITH THE MICROBIAL COMMUNITY

## 6.1 RESULTS

The mixed culture was established in a 1l fermenter (LH Engineering series 500 type) and adapted to tolerate the presence of 36DCPA in the growth medium as described in Chapter 5. When steady states had been achieved, in terms of absorbance, the fermenter contents were harvested and assayed for the following parameters: viable count, absorbance at 600nm, percentage of component organisms, oxygen uptake, concentration of free chloride ions, concentration of 36DCPA and the activity of picolinate 6-hydroxylase. The assays were performed at a series of dilution rates from $0.02h^{-1}$ to $0.32h^{-1}$. The parameters were studied to determine the overall behaviour of the individual community and to try to understand the interactions, if any, between the species present in the community.

### 6.1.1 Biomass in the Fermenter

Biomass was measured in terms of absorbance at 600nm and viable count $ml^{-1}$. Absorbance was measured as described in section 2.6.1 and viable count was measured by plating out 0.1ml of an appropriate dilution (usually $10^{-5}$) of the culture onto nutrient agar.

Figure 6.1 shows the figures for absorbance and viable count at varying dilution rates. The absorbance exhibited a peak at a dilution rate of $0.16h^{-1}$ and declined on either side of this value. At the lowest dilution rate studied ($0.02h^{-1}$) the absorbance was higher than in high dilution rate cultures.

The viable count values also showed a maximum at a dilution rate of $0.16h^{-1}$ and decreased at dilution rates above and below this value. The slope of the increase with increasing dilution rate was much greater than the slope of the decrease with increasing dilution rate.

Figure 6.1 Absorbance at 600nm and viable count (organisms $ml^{-1}$) of the whole mixed population at varying dilution rates.

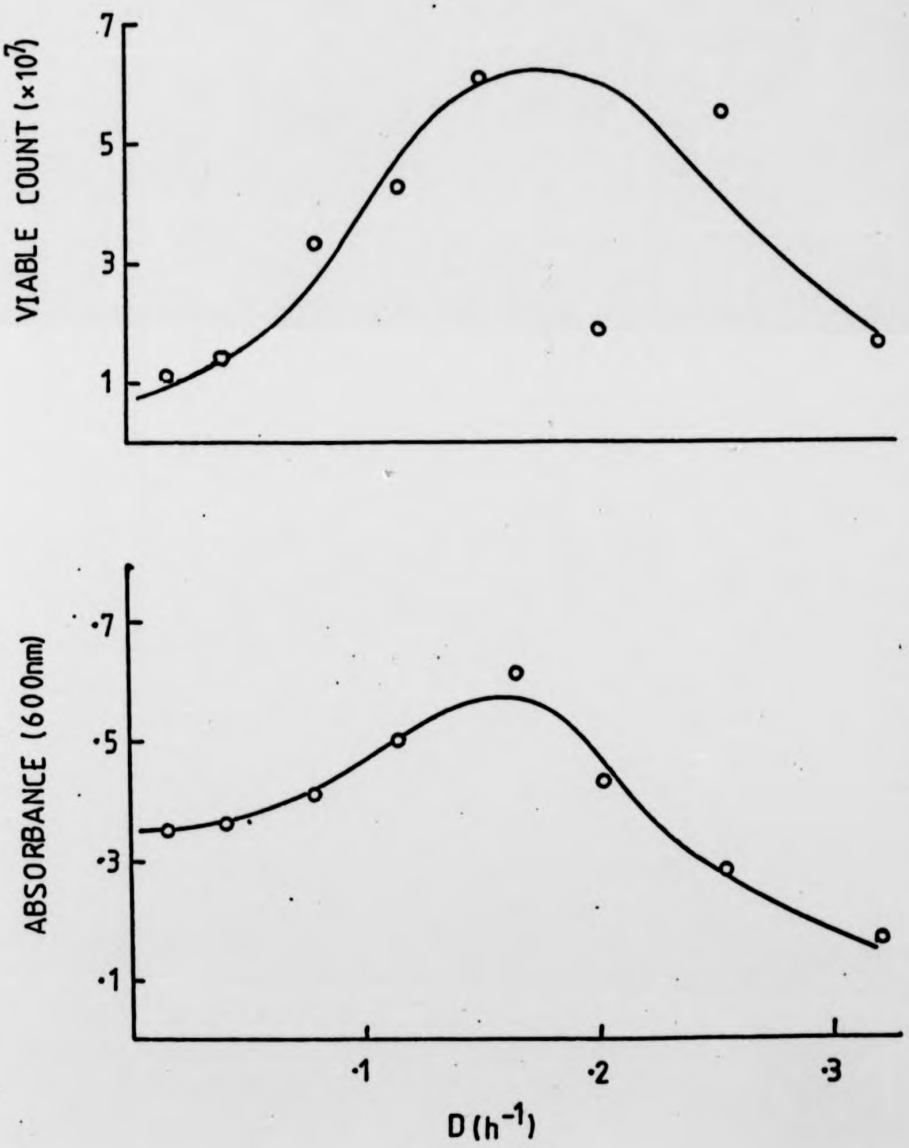
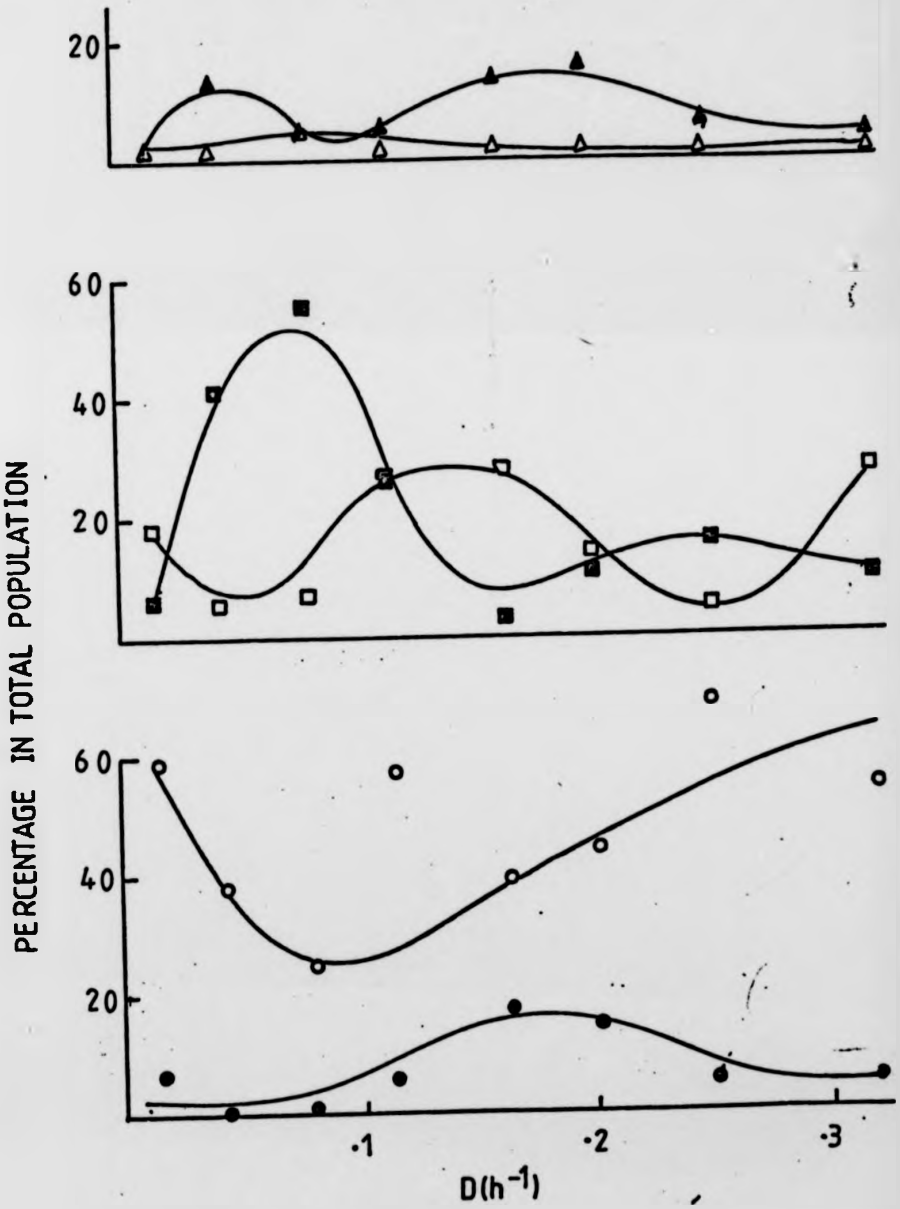

VIABLE COUNT (×10⁷)
7
5
3
1
ABSORBANCE (600nm)
·7
·5
·3
·1
·1
·2
·3
D (h⁻¹)

Figure 6.2 shows the percentage of component organisms of the microbial community at varying dilution rates. Pseudomonas aeruginosa reached a peak of 17% of the total at a dilution rate of $0.16h^{-1}$ but at higher and lower dilutions the population declined to approximately 3%. Alcaligenes faecalis was the most abundant organism in the microbial community. It was present, initially, at 60% of the population but as the dilution rate increased the population declined to a minimum of 25% at a dilution rate of $0.08h^{-1}$. Above this dilution rate the percentage of the organism in the population increased and remained constant at approximately 60%. Since the colonies of A.faecalis were the largest on plates containing picolinate as the sole carbon source, and the organism was the most abundant, it was designated as the major primary utiliser of picolinate in the community. The percentage of the second Alcaligenes sp. in the mixed population varied between 30% and 5%. At a dilution rate of $0.02h^{-1}$ Alcaligenes sp2. comprised 10% of the community. The population declined to a minimum of 6% at a dilution rate of $0.04h^{-1}$ and increased again as the dilution rate of $0.16h^{-1}$. The percentage of organisms declined to 6% at a dilution rate of $0.25h^{-1}$ and rose again to 26% at the highest dilution rate of ($0.32h^{-1}$). The percentage of Bacillus licheniformis remained constant at approximately 10% above a dilution rate of $0.15h^{-1}$. Below this value, however, the percentage rose rapidly to reach a maximum of 54% at a dilution rate of $0.08h^{-1}$ and fell to a minimum of 5% at a dilution rate of $0.02h^{-1}$. The peak of B.licheniformis presence corresponded exactly to the decline in the numbers of A.faecalis at this dilution rate.

Both the Corynebacterium sp and the Rhodococcus sp were minor members of the community. The latter remaining constantly at 1% of the population throughout the entire dilution rate range studied. A small rise in the percentage of Rhodococcus sp was observed at a dilution rate of $0.08h^{-1}$. The Corynebacterium sp exhibited two peaks of 12% and 15% at dilution rates of $0.04h^{-1}$ and $0.202h^{-1}$ respectively. At all other dilution rates the percentage of the population was approximately 5%.

Figure 6.2 The percentage of component organisms present in the mixed population as a function of dilution rate.

● , Pseudomonas aeruginosa
○ , Alcaligenes faecalis
■ , Bacillus licheniformis
□ , Alcaligenes sp.2
▲ , Corynebacterium sp.
△ , Rhodococcus sp.


20
60
40
20
60
40
20
PERCENTAGE IN TOTAL POPULATION
·1
·2
·3
D(h⁻¹)

It was clear that A.faecalis was the dominant organism in the population. Most of the other organisms began to decline when A.faecalis increased upto a maximum as the dilution rate increased. However, all the other organisms were still present. Even at the highest dilution rate examined, $D = 0.32h^{-1}$, none of the organisms was washed out of the community, which illustrated the stability of the mixed culture which had been isolated.

### 6.1.2 Fate of 3,6-Dichloropicolinate in the Fermenter

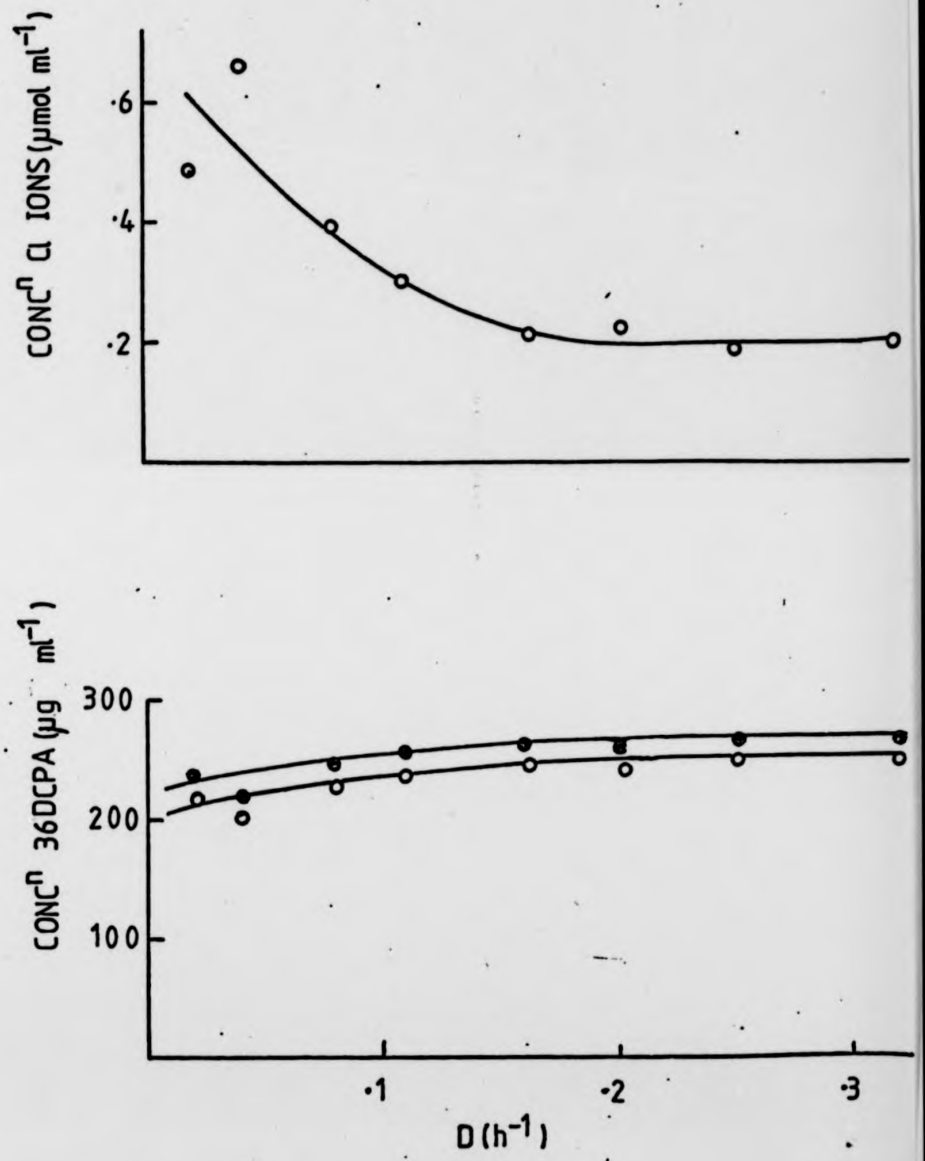
The disappearance of 36DCPA from the fermenter at varying dilution rates was measured by two methods. The actual concentration of 36DCPA was measured directly, as described in section 2.6.4. Also, the concentration of free chloride ions was monitored, as described in section 2.6.3, and from the value of free chloride ions in solution it was possible to estimate the amount of 36DCPA mineralised, as opposed to the amount 36DCPA converted into any other compound that was not detectable as 36DCPA by the gas chromatographic technique.

Figure 6.3 shows the concentration of free chloride ions in the fermenter at varying dilution rates, and the concentration of 36DCPA. These values of 36DCPA have been calculated from the amount of chloride ions released and so represent the amount of 36DCPA remaining that has not been mineralised (but see Figure 6.4 for the concentration of 36DCPA as measued by gas chromatography).

Figure 6.3a shows that the chloride ion concentration was high in low dilution rate cultures but fell steadily to reach a plateau of $0.2\mu mol\ ml^{-1}$ chloride ions in higher dilution rate cultures. The decay curve indicated that 36DCPA was mineralised slowly.

Figure 6.3b shows the amount of 36DCPA in the effluent of the fermenter as calculated by the amount of free chloride ions in solution. The lower curve assumes a residual concentration of chloride ions of $0.2\mu mol\ ml^{-1}$ in the influent medium. The value was chosen since that was the amount of free chloride ions in the effluent at the highest dilution rate of $0.32h^{-1}$. At this dilution rate it was assumed that there was no breakdown of

Figure 6.3 (a) The concentration of free chloride ion in the effluent of the fermenter at varying dilution rates.
(b) The amount of 36DCPA remaining in the effluent when calculated from the amount of chloride ions. (●) asumes no residual chloride in influent medium. (○) assumes a residual chloride concentration of 0.2$\mu$mol $ml^{-1}$ in influent medium (see text for details).


CONCⁿ Cl IONS (μmol ml⁻¹)
·6
·4
·2
CONCⁿ 36DCPA (μg ml⁻¹)
300
200
100
·1
·2
·3
D (h⁻¹)

36DCPA on gas chromatographic evidence (see Figure 6.4). Both figures show that there was some mineralisation of 36DCPA at lower dilution rates and that a plateau was reached above a dilution rate of $0.15h^{-1}$ where no 36DCPA was degraded. At a dilution rate of $0.02h^{-1}$ 26.4% of the 36DCPA added was mineralised (or 15.1% if it was assumed that there was some residual chloride ions in the influent medium). That was equivalent to 70µg $ml^{-1}$ of 36DCPA mineralised.

Figure 6.4 shows the concentration of 36DCPA remaining in the effluent of the fermenter, as measured by gas chromatography, at varying dilution rates. The curve exhibited a plateau above which no 36DCPA was degraded (at dilution rates greater than $0.1h^{-1}$). Below this value the concentration of 36DCPA decreased as the dilution rate decreased. At the lowest dilution rate of $0.02h^{-1}$, the concentration of 36DCPA in the effluent was 160µg $ml^{-1}$. A maximum of 265µg $ml^{-1}$ of 36DCPA was added to the influent medium which indicated that approximately 100µg $ml^{-1}$ of 36DCPA was chemically altered at this dilution rate; that is 38% of the 36DCPA added. This value, when compared with the value for 36DCPA mineralisation calculated from chloride release (26.4%) may have indicated that there was some 36DCPA that was chemically altered but did not release any of its chloride ions (10% or 2.65µg $ml^{-1}$).

These results indicated that at low dilution rates the herbicide 36DCPA was degraded slowly by the microbial community isolated from soil described in Chapter 5.

### 6.1.3 Oxygen Uptake by the Microbial Community

Oxygen uptake was measured in a Rank oxygen electrode as described in section 2.9. The measurements were performed in order to establish whether 36DCPA caused uptake of oxygen and hence indicated aerobic metabolism of the herbicide. Initially the endogenous rate of oxygen uptake was measured, that is, the rate of uptake when no energy source was present. This value was subtracted from the rate of oxygen uptake measured when a carbon source was present, giving the actual amount of oxygen taken up by the organisms due to the presence of the energy source.

Figure 6.4 Concentration of 36DCPA remaining in the effluent of the fermenter, measured by gas chromotography, at varying dilution rates.

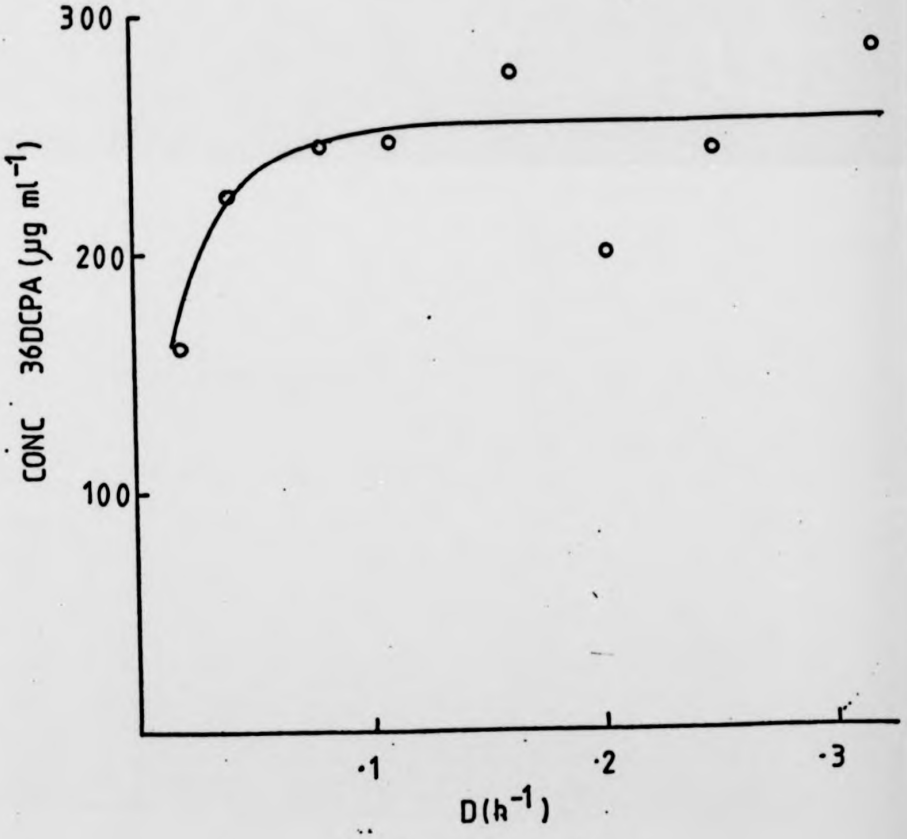
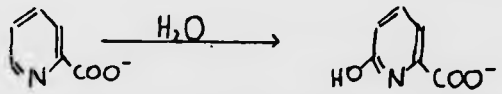

300
200
100
CONC 36DCPA (µg ml$^{-1}$)
·1
·2
·3
D(h$^{-1}$)

Figure 6.5 shows the uptake of oxygen by the mixed microbial population due to picolinate and 36DCPA (both at a concentration of 0.5g carbon $l^{-1}$). At the lowest dilution rate of $0.02h^{-1}$ the amount of oxygen taken up was 1.2mmol $O_2$ taken up (mg dry wt)$^{-1}$ $h^{-1}$ due to picolinate. The rate of oxygen uptake increased rapidly to 9.5mmol $O_2$ taken up (mg dry wt)$^{-1}$ $h^{-1}$ at a dilution rate of $0.04h^{-1}$. At increasing dilution rates the rate of oxygen uptake exhibited an exponential decay curve. When 36DCPA was used as the source of energy the trends were very similar except that the actual value of oxygen uptake was approximately half of that with picolinate at all dilution rates studied.

### 6.1.4 Activity of Picolinate 6-Hydroxylase in the Fermenter

The enzyme that was responsible for the initial attack on the picolinate molecule was picolinate 6-hydroxylase. This enzyme performs the reaction:




that is, a hydroxylation at the 6 position. 6-hydroxypicolinate has a characteristic absorption spectra in the ultra violet range (Dagley & Johnson, 1963). The activity of the enzyme was measured by the method described in section 2.8.

Figure 6.6 shows the activity of the enzyme at varying dilution rates. Two values are shown, the lower was calculated directly from the experimental data when picolinate was added to the cell-free system at a final concentration of 0.557mM. The upper curve was calculated from Lineweaver-Burk plots as V max. The activity had a maximum of approximately 97μmol picolinate hydrolysed (mg protein)$^{-1}$ $h^{-1}$ at a dilution rate of $0.16h^{-1}$ and declined both above and below this dilution rate. The increase in enzyme activity at a dilution rate of $0.32h^{-1}$ may have been due to a wall growth on the fermenter vessel which would support organisms growing at a lower growth rate than in the liquid medium hence effectively reducing the overall growth rate of the population.

Figure 6.5 Oxygen uptake by the microbial population with picolinate (●) and 36DCPA (○) as the source of energy. (Both at a concentration of 0.5g carbon $l^{-1}$).

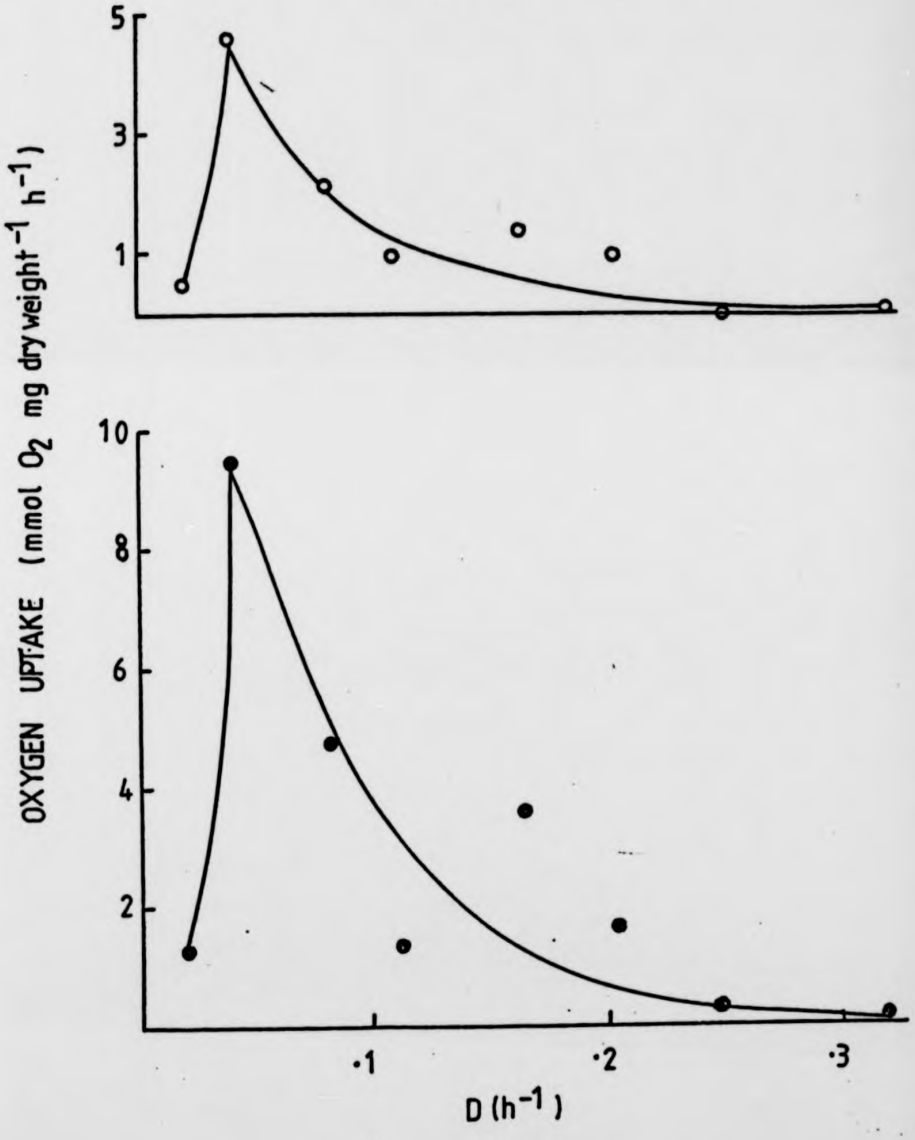

OXYGEN UPTAKE (mmol $O_2$ mg dry weight$^{-1}$ h$^{-1}$)
5
3
1
10
8
6
4
2
·1
·2
·3
D (h$^{-1}$)

Figure 6.6 Activity of the enzyme picolinate-6-hydroxylase at varying dilution rates. (○) value calculated from Lineweaver-Burk plots. (●) value at 0.557 mM picolinate.

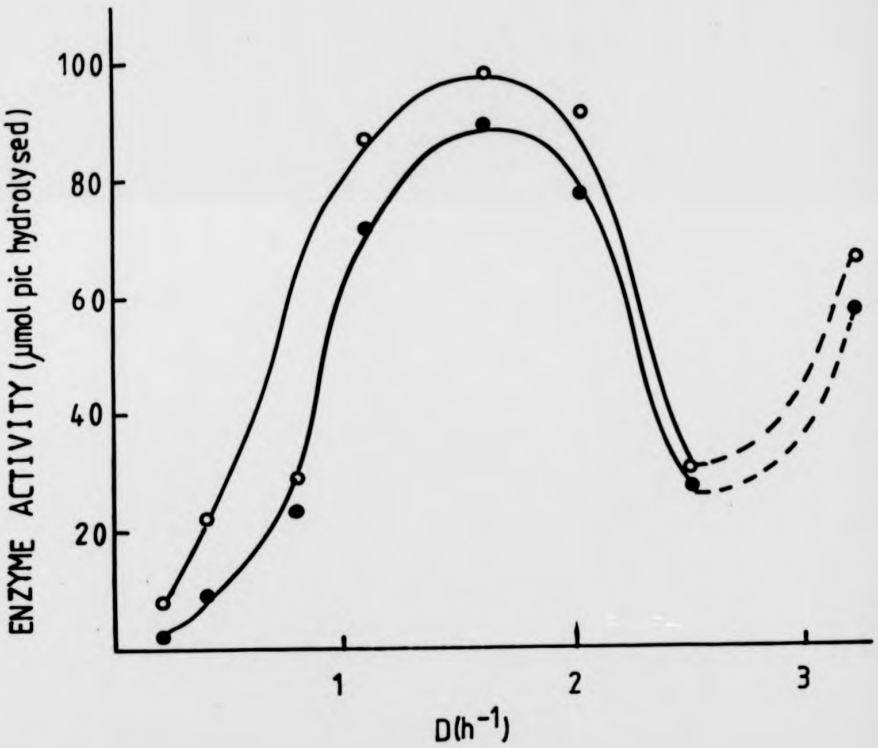

ENZYME ACTIVITY (µmol pic hydrolysed)
100
80
60
40
20
1
2
3
D(h⁻¹)

Figure 6.7 shows the activity of the enzyme at varying initial substrate concentrations at the optimum dilution rate. The plateau region of the curve at high substrate concentrations indicated that the enzyme exhibited first-order reaction kinetics.

Figure 6.8 shows the same data on a Lineweaver-Burk plot of 1/S vs 1/V for the enzyme. The linear relationship demonstrated, again, first-order reaction kinetics.

Figure 6.9 shows the enzyme activity at varying pH at the optimum dilution rate. The activity exhibited two peaks, one at pH 7.25 and one at pH 8.5. The enzyme activity rose from pH 5.5 at a steady rate to 31μmol picolinate hydrolysed (mg protein)$^{-1}$ h$^{-1}$ at pH 7.25, fell slightly and rose to a maximum of 33μmol picolinate hydrolysed at pH 8.5. The activity fell rapidly as pH increased above 8.5.

The inhibition of the enzyme by 36DCPA and 6-chloropicolinate (6MCPA) was studied. 6MCPA did not inhibit the enzyme but 36DCPA did act as an inhibitor.

Figure 6.10 shows the Lineweaver-Burk plot for the inhibition by 36DCPA using concentrations of 0, 0.302, 0.906 and 1.509mM. Linear regression analysis was carried out using the Newton-Raphson method, by the program outlined in appendix 1, using the formulae

$$\text{slope} = \frac{(n.\sum(nxy)) - (\sum(nx).\sum n)}{n\sum(nx^2) - (\sum(nx)^2)}$$

$$\text{intercept} = \frac{(n.\sum(ny).\sum(nx^2)) - (\sum(nx).\sum(nxy))}{n\sum(nx^2) - (\sum(nx)^2)}$$

The Michaelis-Menton constant, Km, value for picolinate was calculated, since the intercept of the 1/S axis with the line I = 0 was equal to $-\frac{1}{Km}$; that is Km = 0.0838mM which is equivalent to $8.4 \times 10^{-5}$M.

Figure 6.7 Enzyme activity (V) at varying substrate concentrations (S) to exhibit 1st order kinetics.

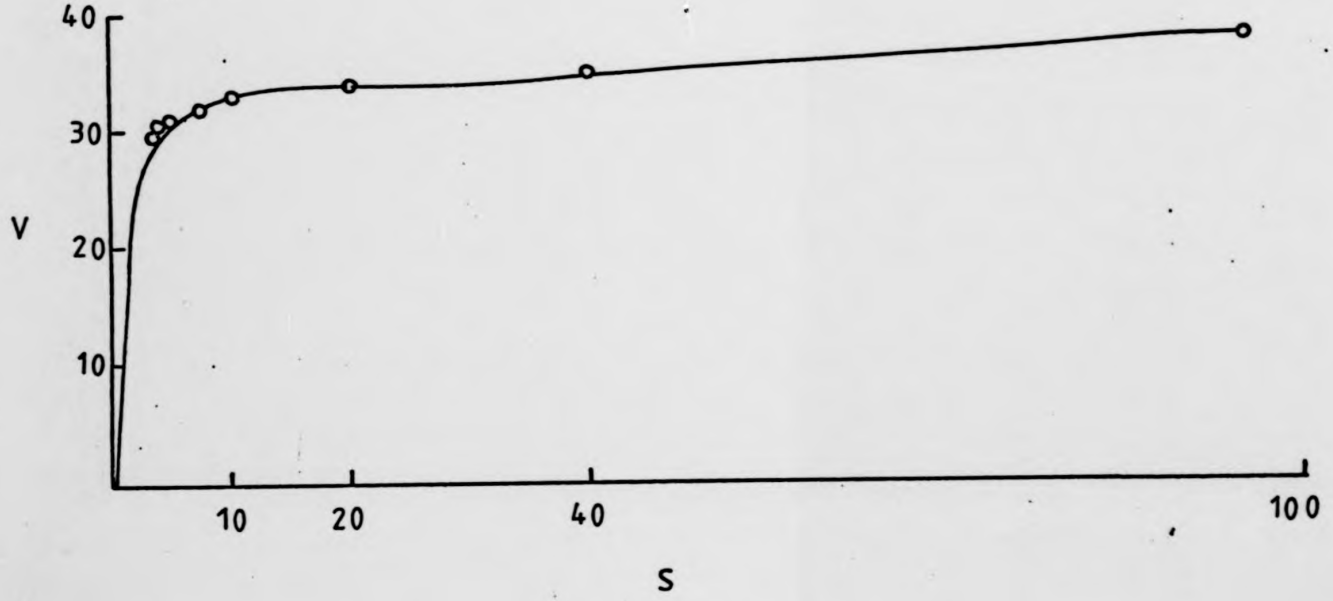

40
30
20
10
V
10
20
40
100
S

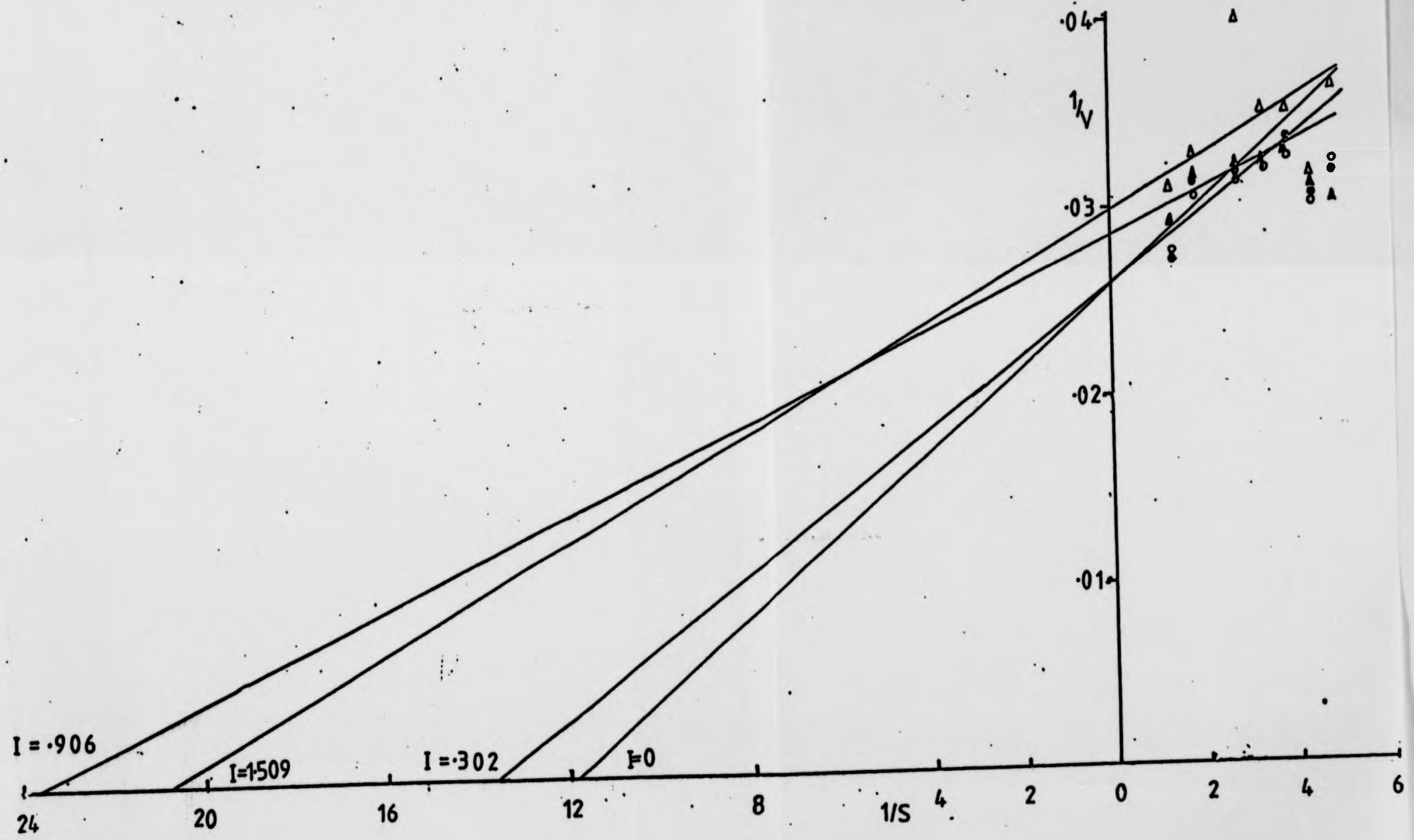
Figure 6.8 Lineweaver-Burk plot showing first order reaction kinetics of picolinate-6-hydroxylase.

1/V
·04
·03
·02
·01
0
1
2
3
4
1/S

Figure 6.9 Activity of picolinate-6-hydroxylase at varying pH. Cells were grown before harvesting at the optimum dilution rate in a chemostat.

ENZYME ACTIVITY (μmol pic hydrolysed mg protein⁻¹ h⁻¹)
0
10
20
30
PO4
TRIS-MALEATE
GLYCINE-NaOH
5·5
6
7
8
9
10
10·5
pH

Figure 6.10 Lineweaver-Burk plot of inhibition of picolinate-6-hydroxylase by 36DCPA. Inhibitor concentration equals 0mM (●), 0.302mM (○), 0.906mM (▲), 1.509 (△).


·04
1/V
·03
·02
·01
I = ·906
I=1509
I = ·302
I=0
24
20
16
12
8
1/S
4
2
0
2
4
6

The apparent inhibition constant, $K_I$ was similarly calculated, since the intercept of any line with the 1/S axis is $-\frac{1}{Km}(1 + \frac{i}{K_I})$. The values calculated for $K_I$ were 2.059mM. 0.914mM and 1.987mM using inhibitor concentration of 0.302, 0.906 and 1.509mM respectively. The average value of the apparent $K_I$ was 1.65mM. However it should be noted that the scatter of these points means that the calculations of $K_I$ are very unreliable. This data scatter could be attributed to enzyme multiplicity.

## 6.2 Discussion

Pure cultures growing on a single source of carbon and energy, generally, exhibit growth characteristics in continuous culture as described in Pirt (1975). In the experiments described in this chapter there was a marked difference to this accepted standard. There was a peak in the biomass at $D=0.16h^{-1}$. The reasons for this peak could be related to the nature of the mixed culture and its interactions, and the presence of two carbon sources.

One more factor that may have contributed to the peak of biomass was the presence of wall growth at higher dilution rates, and this will be discussed later in relation to the activity of the hydroxylase available for comparison with these results. Senior et al (1976) did not specify the biomass concentrations at varying dilution rates and much of the work on recalcitrant molecules and co metabolism of such molecules does not describe any continuous culture experiments. Harrison, Wilkinson Wren & Harwood (1977) have described a mixed culture as a basis for single cell production from $C_1$ compounds, and this showed the standard curve of biomass versus dilution rate.

The viable count values coincide with the biomass values indicating an approximately linear relationship between biomass by weight and biomass by numbers as might be expected.

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The viable count values coincide with the biomass values indicating an approximately linear relationship between biomass by weight and biomass by numbers as might be expected.

The percentage of component organisms however does not fluctuate with dilution rate. There has been little work where the component populations have been isolated, identified and monitored with which to compare these results. Cremieux et al (1977) measured the percentage of the component organisms of their mixed culture only at a single dilution rate ($0.34h^{-1}$) and Van Gemerden (1974) studied the effect of light on the steady state values of the component populations at a dilution rate of $0.011h^{-1}$.

The marked decrease of the primary utiliser A. faecalis at D = .08, is mirrored by a large increase in the population of B. licheniformis. This could be due to competition between these organisms for a particular growth factor. The reason for A. faecalis not falling to a much lower level being that its presence is necessary for initial attack on the picolinate molecule.

The peaks of Ps. aeruginosa and Alcaligenes sp.2 show competition for and maximum utilisation of the picolinate substrate, along with A. faecalis. It is possible that many other factors, including all the different parameters mentioned in section 1.5, may affect the varying proportions of these organisms, and indeed all the organisms in the mixed culture.

The exponentially decreasing curve of free chloride ions in the fermenter (figure 6.3a) may be related to the residence time of the compound in the fermenter in that at low dilution rates the organisms have a relatively longer time during which metabolic attack of the 36DCPA may occur than at high dilution rates.

The values calculated for the amount of 36DCPA broken down are comparable for the two methods used. The apparent discrepancy between the G/C method and the chloride method may be due to the accumulation of 3-chloro-6-hydroxypicolinate since this compound has been found by other workers (Pik et al., 1977; Swann, Regoli, Comeaux and Laskowski, 1976). However, Houghton and Cain (1973) found that pyridinediols accumulated upon the degradation of pyridine, notably the 2-5-pyridinediol, which would require the release of both chloride ions to form in case of 36DCPA (ignoring the acid group). Grant and Al-Najjar (1976) observed a similar activity by a soil bacterium growing on quinoline. The organisms first action was hydroxylation of the carbon molecule next to the nitrogen molecule, followed by attack diagonally across the ring structure (in this case the two rings of quinoline).

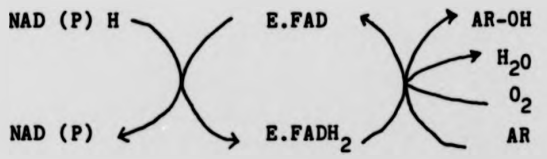
As stated in section 6.1.2 the results indicated that, at low dilution rates 36DCPA was degraded slowly by the mixed population. The nature of the attack on the compound was not determined but it may have been a cometabolic attack since the results described in chapter 4 indicated that no breakdown of 36DCPA occurs either with or without a carrier substrate. It may be that the enzymes specified for the breakdown of picolinate performed a cometabolic attack due to the chemical similarity of the two compounds.

A generalised equation for the action of an aromatic hydroxylase is given in equation 6.1 (Mahler and Cordes, 1971).

$$Ar + O_2 \xrightarrow[\text{equivalents}]{\text{Reducing}} ArOH + H_2O \qquad 6.1$$

The rate of oxygen uptake, therefore is a direct measure of the rate of degradation of an aromatic compound attacked by this type of enzyme. The results shown in figure 6.4 would seem to indicate that at the maximum activity studied, i.e. D = .04, 36DCPA is degraded at half the rate of picolinate approximately. (4.6 and 9.4 mmol $O_2$/mg dry w/hr respectively). These results are clearly a discrepancy on the results obtained from chloride release and gas chromatographic studies, since they indicate a very much decreased rate of degradation.

The discrepancy may be explained by studying equation 6.1 further. In order to produce the reducing equivalents the electron transport chain is involved and a generalised scheme is shown in equation 6.2 (Mahler and Cordes, 1971; Dagley, 1975).




6.2

It has been found in certain cases that the action of the electron transport chain can be uncoupled from the oxidation of the aromatic molecule resulting in oxygen uptake being demonstrable but the hydroxylated aromatic was not detectable. (Ribbons and Ohta, 1970; Ribbons, Ohta and Higgins, 1970).

Clearly, although oxygen uptake is an indication of the rate of degradation of 36DCPA, the results must not be studied in isolation but should be used in conjuction with other results due to the possible uncoupling of oxygen use from hydroxylation.

The enzyme picolinate 6-hydroxylase shows the characteristics of a catabolic enzyme as expounded by Clarke and Lilly (1969). This type of enzyme has a low activity at low dilution rates, increasing to a maximum activity at intermediate dilution rates and decreasing to a low activity at high dilution rates.

At low dilution rates the enzyme activity is low due to small amounts of substrate-cum-inducer concentration. As the substrate concentration increases the enzyme activity increases due to a higher level of induction. As the dilution rate increases further the production of enzyme, and hence the activity is cell free extract, falls due to repression by one or more catabolites.

The increase in enzyme activity at very high dilution rates may have been due to wall growth in the fermenter decreasing the overall growth rate of the fermenter contents. Wilkinson and Hamer (1974) found that wall growth had a significant effect on the growth characteristics of mixed cultures growing on methane. They hypothesised that, because their system was oxygen limiting and the wall layer was likely to be more than one cell thick, growth rate would be decreased in the wall growth as opposed to growth in the body of the fermenter.

They also suggested that the proportion of organisms from each species present in the mixed culture may not have been the same, in the wall growth, as in the body of the fermenter. This is an interesting observation that was not studied in the wall growth observed in the growth described in this thesis.

The pH profile of picolinate 6-hydroxylase in cell free extracts from the mixed population shows multiple peaks (Figure 6.8). The reason for this may be due to the complex nature of the cell free extract being examined, since it was derived from a mixed culture. It would seem to imply that the hydroxylase activity from the primary utilisers had different optimum pH's. This, in turn, may explain why the colonies of Alcaligenes sp2. were very much smaller on phosphate buffered agar than those of A.faecalis after an identical incubation period. It may also indicate enzyme multiplicity. Mixed cultures of varying constituents were examined to try to establish this point and these results are detailed in chapter 7.

It is interesting to note that 6-monochloropicolinate did not inhibit picolinate 6-hydroxylase at the concentrations studied whereas 36DCPA did inhibit the enzyme. This is evidence that the biological recalcitrance of 36DCPA stems from the present of the chloride group at the 3$^{a}$ position. It could mean that after the initial hydroxylation, the attack of an hydroxide group diagonally opposite the first hydroxylation was energetically unfavourable, and so normal formation of the diol was inhibited and hence ring cleavage was inhibited. It would be interesting to study the action of the hydroxylase against a disubstituted picolinic acid that had either its substituted group in a different position, or a more electropositive group at the 3 position, say a methyl group.

The inhibition of picolinate-6-hydroxylase by 36DCPA exhibited mixed inhibition. This mixed inhibition may have been due to the presence of more than one organism in the mixed culture, and hence different enzymes in the cell extract. This would point to very different enzymes being induced in the populations present in the mixed culture. In conjunction with the pH curve the results may indicate multiple enzyme production since there is a peak in activity at basic, neutral and acidic values of the pH curve.

CHAPTER 7

# STUDIES ON THE ENZYMOLOGY OF PICOLINATE BREAKDOWN IN VARIOUS MIXED AND PURE CULTURES

## 7.1 Results

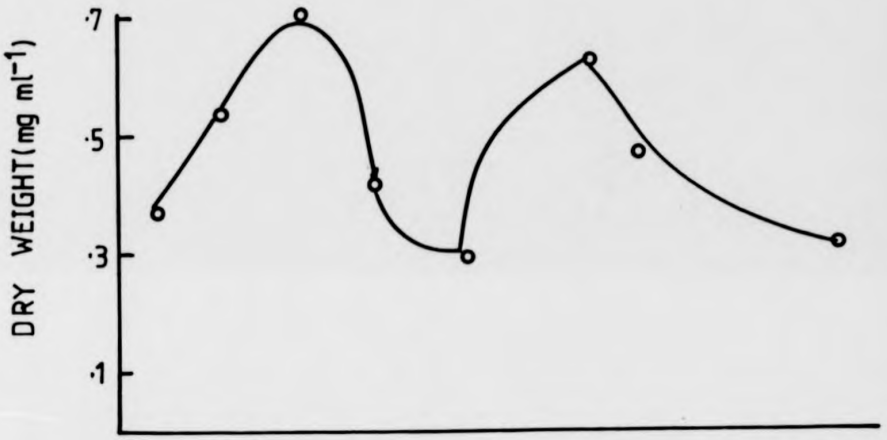
Growth of various mixed cultures derived from the originally isolated mixed culture were examined in a continuous flow culture in an attempt to shed light on the mechanism of interaction of the community, the mechanism of stability of the community and the mechanism of toleration to 36DCPA. The parameters measured in each case were absorbance at 600nm, dry weight, percentage of individual organisms in the community and the activity of picolinate 6-hydroxylase. A faecalis was examined in pure culture since it was the predominant primary utiliser of picolinate (see section 5.1.2).

### 7.1.1 Alcaligenes faecalis in Pure Culture

The growth of the A.faecalis strain isolated in the mixed culture was studied with either picolinate as the sole carbon source at a concentration of 0.5g carbon $l^{-1}$ or with picolinate and 36DCPA as the combined carbon source at a concentration of 0.5 and 0.1g carbon $l^{-1}$ respectively. The organism was cultivated in a fermenter of the "Quickfit" type and prepared for assays as described in section 2.

Figure 7.1 shows the biomass concentration in the fermenter in terms of dry weight and absorbance at 600nm when the organism was grown on picolinate alone. Both parameters showed an initial increase as the dilution rate increased followed by a fall and subsequent rise at medium dilution rates. This could be attributed to steady states not being achieved although the same tests for steady states were used at each dilution rate. It was noted, however, that the relationship between dry weight and absorbance was not linear which may indicate a difficulty in estimating dry weight correctly and that care must be taken when estimating biomass solely in terms of absorbance.

Figure 7.1 Absorbance at 600nm and dry weight of A.faecalis growing in chemostat culture on picolinate (at a concentration of 0.5g carbon $l^{-1}$) as sole source of carbon and energy.


DRY WEIGHT(mg ml$^{-1}$)
·7
·5
·3
·1

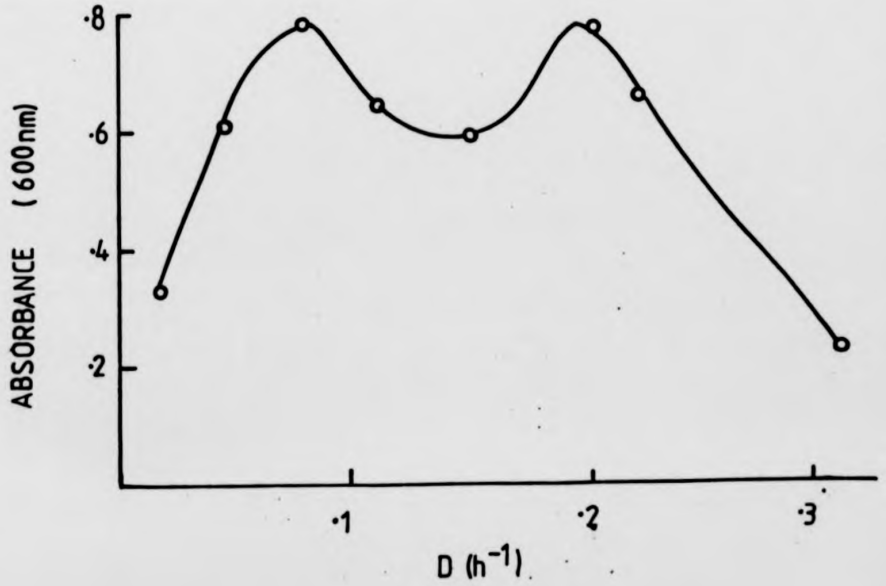

ABSORBANCE (600nm)
·8
·6
·4
·2
·1
·2
·3
D (h$^{-1}$)

Figure 7.2 shows the activity of the enzyme picolinate 6-hydroxylase in cell-free extracts of A.faecalis grown on picolinate alone as the sole source of carbon at varying dilution rates. The optimum activity of 125 µmol hydrolysed (mg protein)$^{-1}$ h$^{-1}$ occurred at a dilution rate of 0.11h$^{-1}$ and declined with both increasing and decreasing growth rates.

Figure 7.3 shows the absorbance of 600nm and the dry weight of a culture of A.faecalis growing on the mixed substrate at varying dilution rates. The comparison of these results with Figure 7.1 shows the effect that 36DCPA had on the growth of A.faecalis in continuous flow culture. There was no plateau effect in either of the parameters studied. The dry weight increased slowly to a maximum of 0.55mg ml$^{-1}$ at a dilution rate of 0205h$^{-1}$ and decreased with increasing dilution rate. The absorbance showed a peak at a dilution rate of 0.255h$^{-1}$ of 0.81 and decreased above and below this value.

Figure 7.4 shows the activity of picolinate 6-hydroxylase extracted from A.faecalis grown on the mixed substrate at varying dilution rates. The activity reached a maximum of 130 µmol picolinate hydrolysed (mg protein)$^{-1}$ h$^{-1}$ at a dilution rate of 0.08h$^{-1}$. At higher dilution rates the activity declined to a minimum of 51 µmol picolinate hydrolysed. At dilutions rates less than the optimum the activity also declined.

At the optimum dilution rate for maximum enzyme production (D = 0.11 h$^{-1}$) the pH profile of the enzyme was studied. The inhibition of the enzyme by 36DCPA was also measured in extract from organisms grown at this dilution rate.

Figure 7.5 shows the pH profile of picolinate 6-hydroxylase activity extracted from A.faecalis in pure culture. The curve has three main peaks at pH's 6.25, 7.0 and 8.5. Below pH 6.25 the activity rapidly decreased to a value of 70 µmol picolinate hydrolysed. Troughs can be observed between three peaks and above pH 8.5 the activity declined to a minimum of 24 µmol picolinate hydrolysed. It can be assumed that there was no effect of the different types of buffer used on the system since at the pH's where buffers changed duplicate results from two different buffers showed no change in the enzyme activity.

Figure 7.2 Picolinate 6-hydroxylase activity of cell-free extracts of A.faecalis growing in continuous culture with picolinate as the sole source of carbon and energy. (At a concentration of 0.5g carbon $l^{-1}$).

(●) activity at 1.79 mM picolinate

(○) maximum activity from Lineweaver-Burk plots.

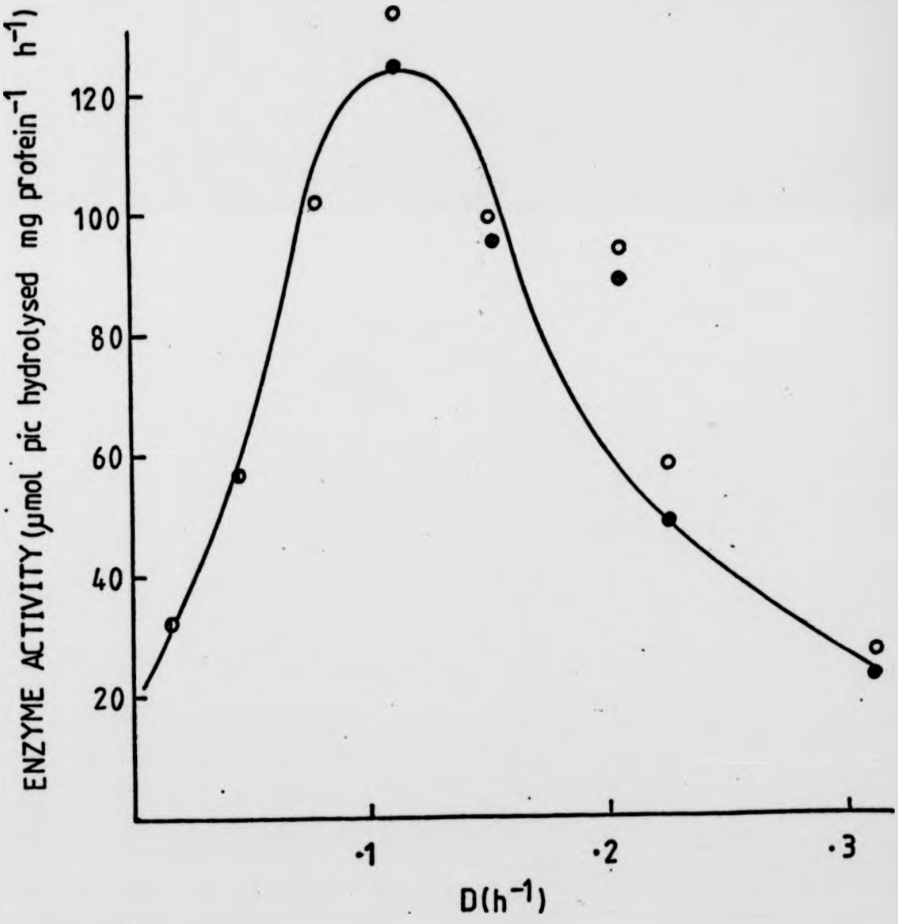
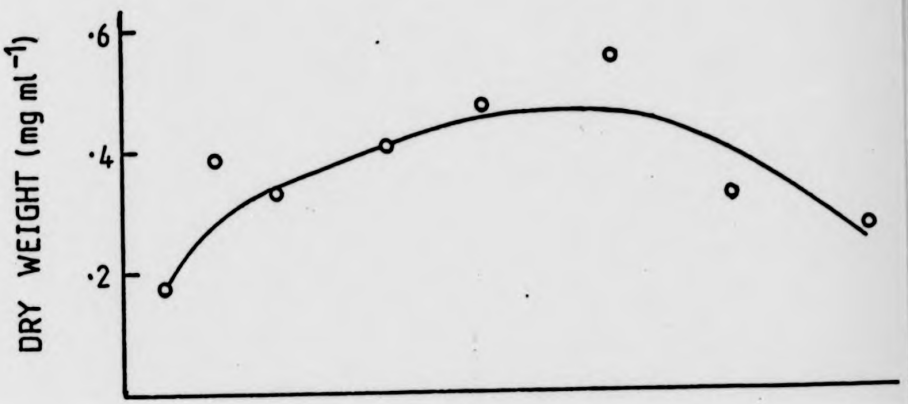

ENZYME ACTIVITY (µmol pic hydrolysed mg protein$^{-1}$ h$^{-1}$)
120
100
80
60
40
20
·1
·2
·3
D(h$^{-1}$)

Figure 7.3 Absorbance at 600nm and dry weight (mg $ml^{-1}$) of A.faecalis growing in chemostat culture with picolinate and 36DCPA as the source of carbon (0.5 and 0.1 g carbon $l^{-1}$ respectively).


DRY WEIGHT (mg ml$^{-1}$)
·6
·4
·2

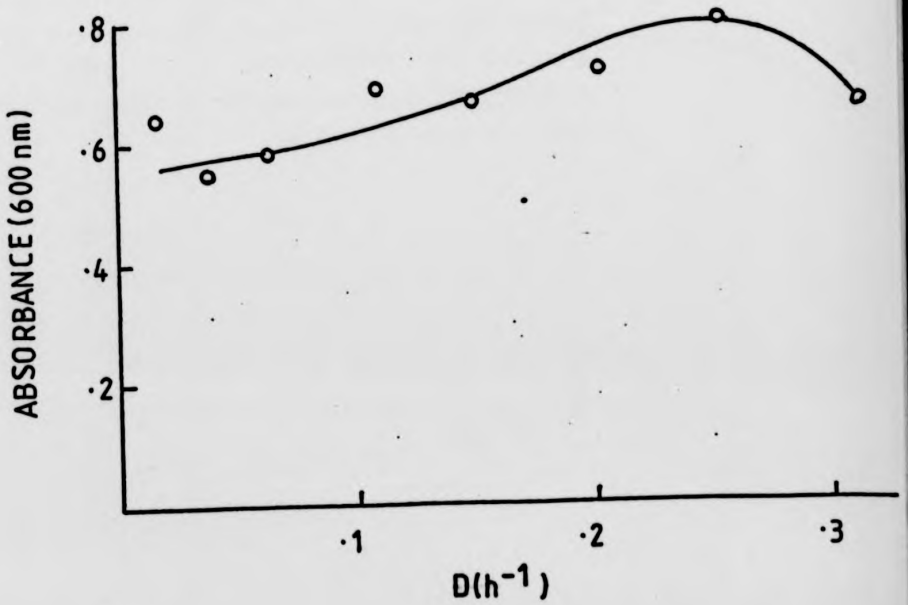

ABSORBANCE (600 nm)
·8
·6
·4
·2
·1
·2
·3
D(h$^{-1}$)

Figure 7.4 Picolinate-6-hydroxylase activity of cell-free extract of A.faecalis growing in chemostat culture with picolinate and 36DCPA as the source of carbon (at a concentration of 0.5 and 0.1 g carbon $l^{-1}$ respectively). (o) activity at 0.558mM picolinate. (●) maximum activity calculated from Lineweaver-Burk plots.

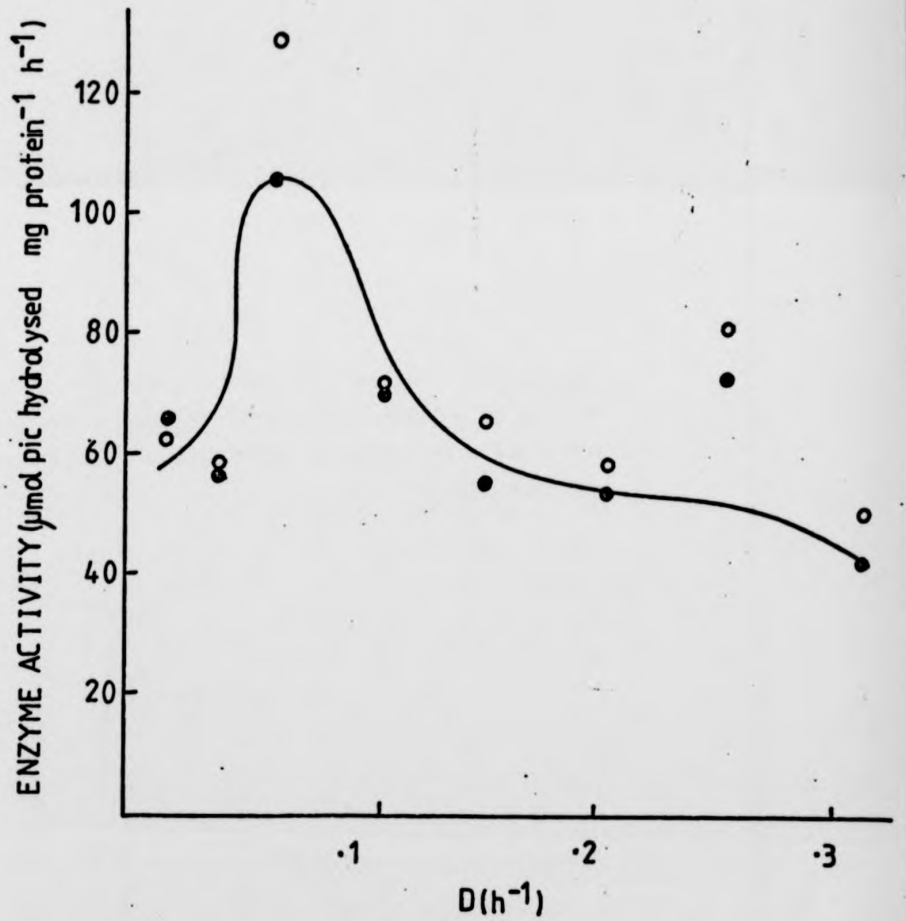

ENZYME ACTIVITY (μmol pic hydrolysed mg protein$^{-1}$ h$^{-1}$)
120
100
80
60
40
20
·1
·2
·3
D(h$^{-1}$)

Figure 7.5 Picolinate 6-hydroxylase activity at varying pH of pure culture of A.faecalis Extract was taken from fermenter operating at the optimum dilution rate.

ENZYME ACTIVITY (μmol pic hydrolysed mg protein⁻¹ h⁻¹)
120
100
80
60
40
20
5·5
6
7
8
9
10
10·5
pH

Table 7.1 shows the inhibitory effect of 36DCPA on picolinate 6-hydroxylase activity at pH 8.5 from A.faecalis growing at a dilution rate of $0.11h^{-1}$. The apparent saturation constant was calculated for picolinate and found to be 0.526 mM. The apparent inhibition constant $K_I$ was calculated to be 0.367 mM by computer analysis of Lineweaver-Burk data.

### 7.1.2 Two Membered Mixed Culture Comprising A.faecalis and Alcaligenes sp2

The two membered mixed culture comprising A.faecalis and Alcaligenes sp2 was studied in continuous culture on the mixed substrate as described in section 2. The inoculum consisted of 100ml of late exponentially growing organisms of each species grown on picolinate alone in closed culture. The organisms were inoculated simultaneously into the fermenter and allowed to grow in closed culture for a further 48h before initiating the flow of fresh medium.

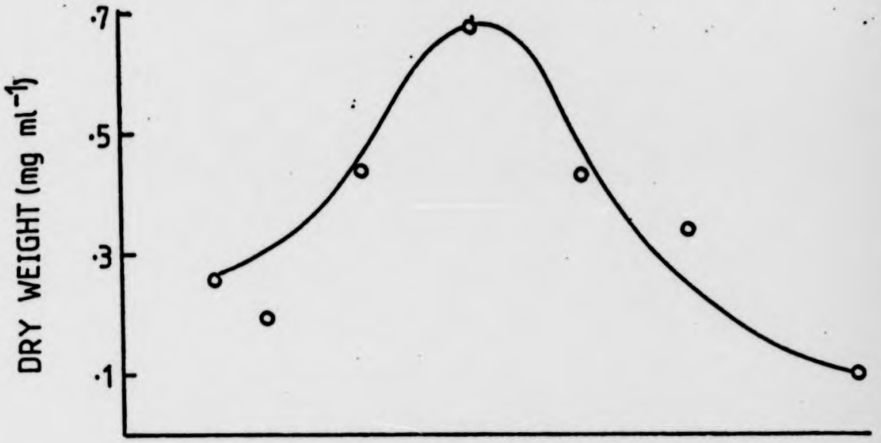
The culture was harvested and prepared for assays as described in section 2. The colonies were identified by morphology alone on picolinate containing agar since colonies of A.faecalis were large and opaque and colonies of Alcaligenes sp2 were small and translucent.

Figure 7.6 shows the absorbance at 600nm and the dry weight of the mixed culture at varying dilution rates. Both curves exhibited a maximum at a dilution rate of approximately $0.16h^{-1}$ and decreased above and below this dilution rate. The mixed culture was maintained as a stable mixed population throughout the course of the experiment.

Table 7.1 Inhibition of picolinate 6-hydroxylase activity at pH 8.5 from A.faecalis growing at a dilution rate of 0.11 $h^{-1}$

| Concn of inhibitor (mM) | Activity at 2.75mM picolinate | %age Activity of inhibited cultures |
|---|---|---|
| 0 | 121.7 | - |
| .302 | 117.9 | 92.5 |
| .906 | 117.9 | 92.5 |
| 1.509 | 113.3 | 89 |

Figure 7.6 Absorbance at 600nm and dry weight (mg $ml^{-1}$) of two species culture comprising A.faecalis and Alcaligenes sp 2 in chemostat culture grown on picolinate and 36DCPA as the source of carbon, at a concentration of 0.5 and 0.1 g carbon $l^{-1}$ respectively.


DRY WEIGHT (mg ml$^{-1}$)
·7
·5
·3
·1

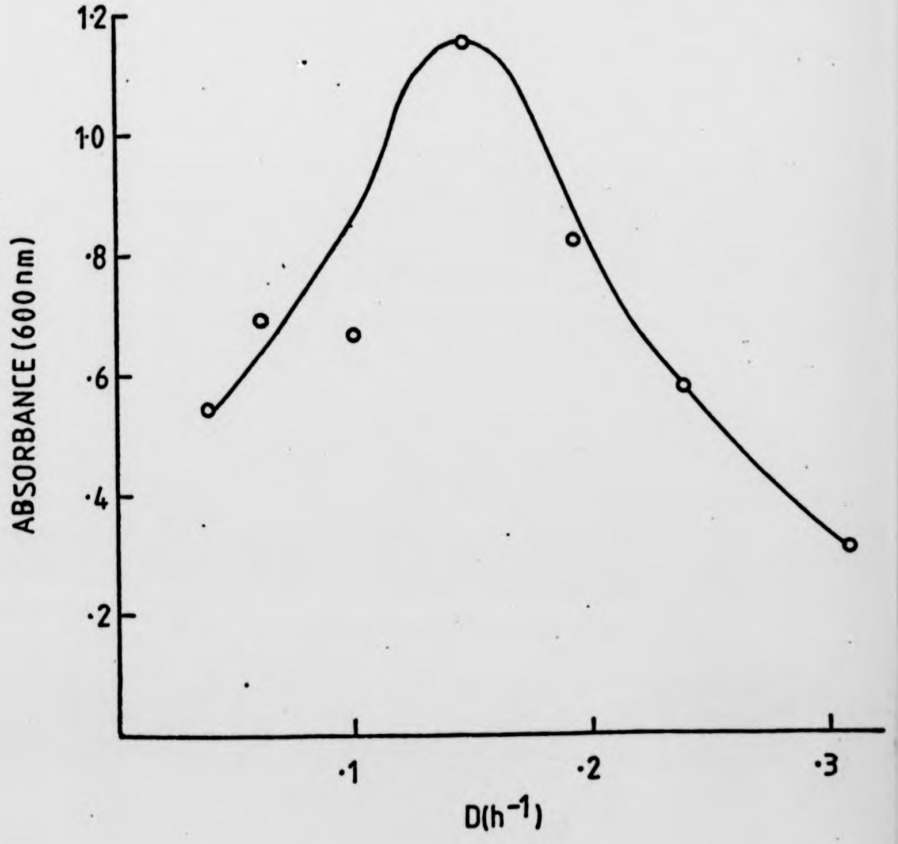

ABSORBANCE (600 nm)
1·2
1·0
·8
·6
·4
·2
·1
·2
·3
D(h$^{-1}$)

Figure 7.7 shows the activity of picolinate 6-hydroxylase in the mixed culture at varying dilution rates. The activity exhibited a maximum of 87 µmol picolinate hydrolysed (mg protein)$^{-1}$ h$^{-1}$ at a dilution rate of 0.076h$^{-1}$. The activity declined rapidly above and below this optimum dilution rate. The high value shown at a dilution rate of 0.31 h$^{-1}$ may have been due to wall growth in the fermenter lowering the actual growth rate.

Figure 7.8 shows the percentage composition of the mixed culture at varying dilution rates. At all dilution rates studied A.faecalis was the predominant organism ranging from 87% at a dilution rate of 0.04h$^{-1}$ to approximately 100% at a dilution rate of 0.31 h$^{-1}$. However, Alcaligenes sp2 was still present in the mixed culture at the highest dilution rate, since, on lowering the dilution rate the percentage of Alcaligenes sp2 returned to its normal level. At the lowest dilution rate studied Alcaligenes sp2 constituted only 13% and this percentage decreased as the dilution rate increased until at a dilution rate of 0.2h$^{-1}$ the organism was present at less than 1% of the culture.

At the optimum dilution rate for enzyme activity the pH profile of the enzyme (or enzymes from both organisms) and the inhibition of the hydroxylase activity by 36DCPA was measured. Figure 7.9 shows the pH profile of picolinate 6-hydroxylase extracted from the mixed culture of A.faecalis and Alcaligenes sp2. At pH 5.5 the activity was 42 µmol picolinate hydrolysed (mg protein)$^{-1}$ h$^{-1}$. The activity rose rapidly to a peak of 86 µmol picolinate hydroysed at pH 6.5, fell slightly and rose to a maximum of 105 µmol picolinate hydrolysed at pH 7.0. Above pH 7.0 the activity fell, slowly at first but more rapidly later, as the pH increased. At pH 10.5 the activity was 26 µmol picolinate hydrolysed (mg protein)$^{-1}$ h$^{-1}$.

Table 7.2 shows the inhibition of picolinate 6-hydroxylase activity by 36DCPA at a dilution rate of 0.076h$^{-1}$. The apparent Km for picolinate and apparent $K_I$ for 36DCPA were calculated from the intercepts of the 1/S axis as described earlier. The apparent Km was found to be 0.065mM and the apparent $K_I$ was found to be 0.39mM. Computer analysis was used for the calculations since scatter on the data made visual line drawing unreliable.

Figure 7.7 Picolinate 6-hydroxylase activity of extract from mixed culture comprising A.faecalis and Alcaligenes sp 2. in chemostat culture grown on picolinate and 36DCPA as the source of carbon at a concentration of 0.5 and 0.1 g carbon $l^{-1}$ respectively. (●) activity at 0.558nM picolinate; (o) maximum activity calculated from Lineweaver-Burk plots.

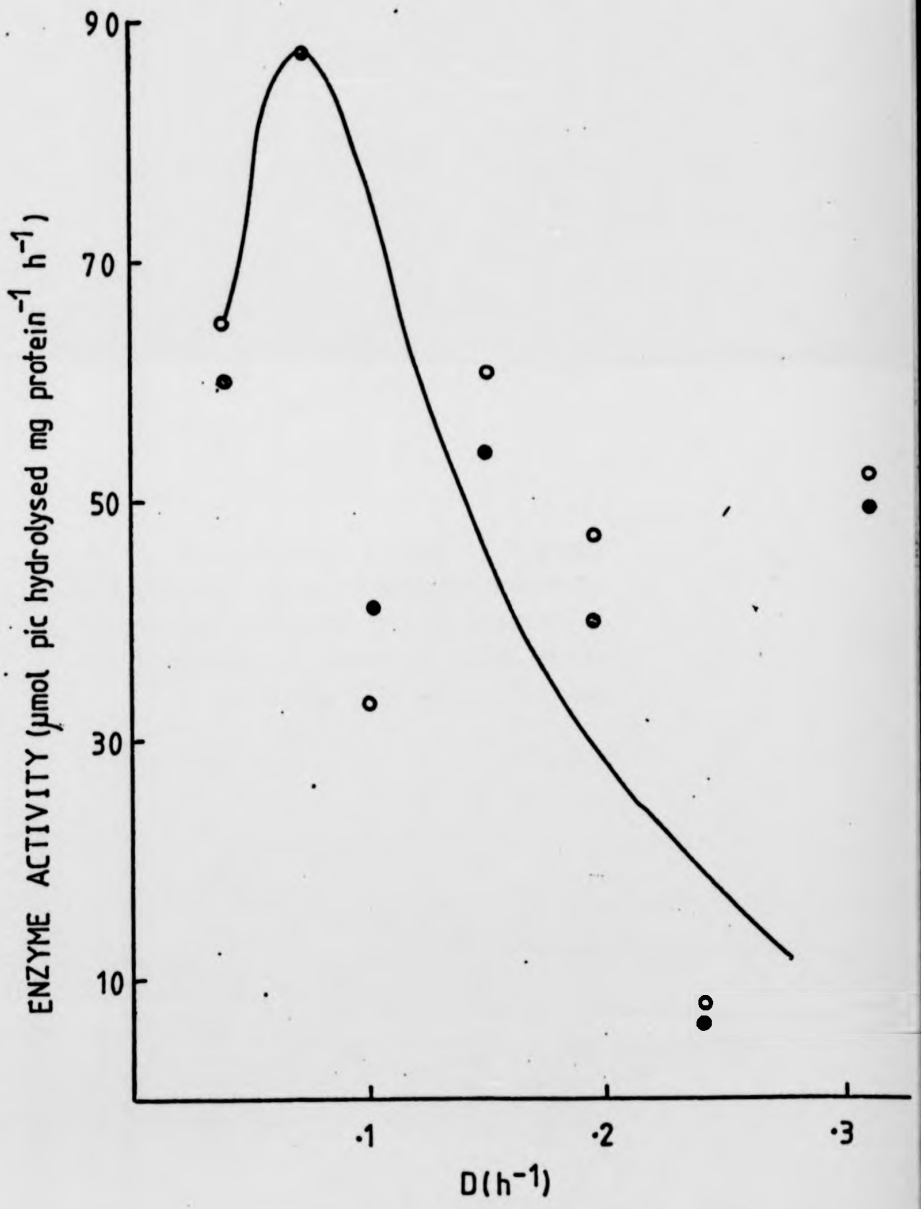

90
70
50
30
10
ENZYME ACTIVITY (µmol pic hydrolysed mg protein$^{-1}$ h$^{-1}$)
·1
·2
·3
D(h$^{-1}$)

Figure 7.8 Species composition of mixed culture comprising A.faecalis (●) and Alcaligenes sp 2. (o) in chemostat culture grown on picolinate and 36DCPA as the source of carbon at a concentration of 0.5 and 0.1 g carbon $l^{-1}$ respectively.

ture
(o)
DCPA
0.5

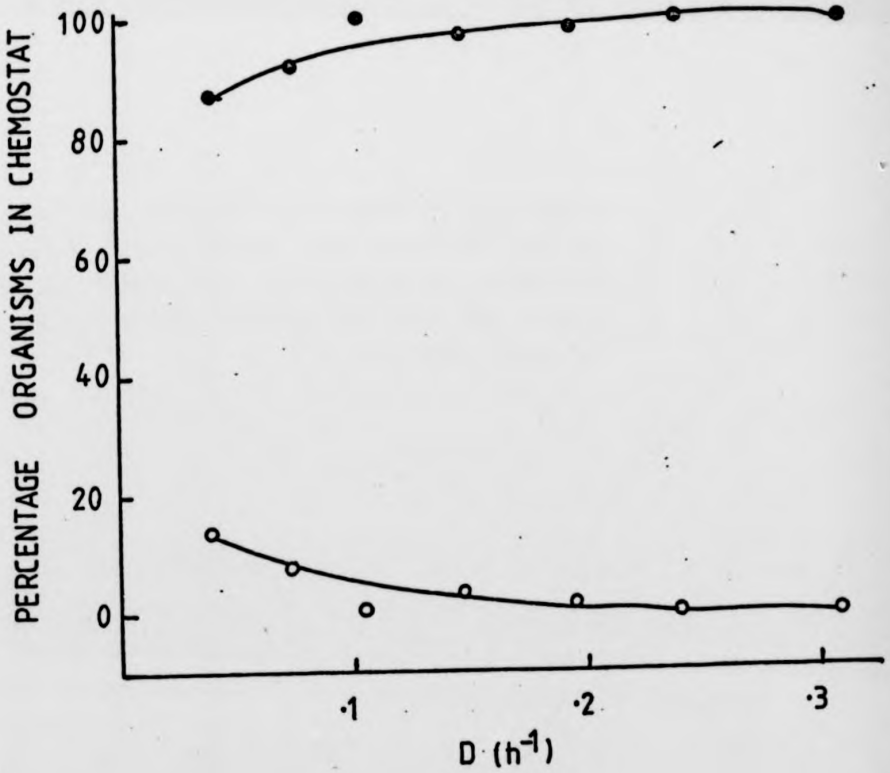


Figure 7.9 Picolinate 6-hydroxylase activity at varying pH from two species mixed culture comprising A.faecalis and Alcaligenes sp 2. Extract was taken from cells growing at optimum D for enzyme production.

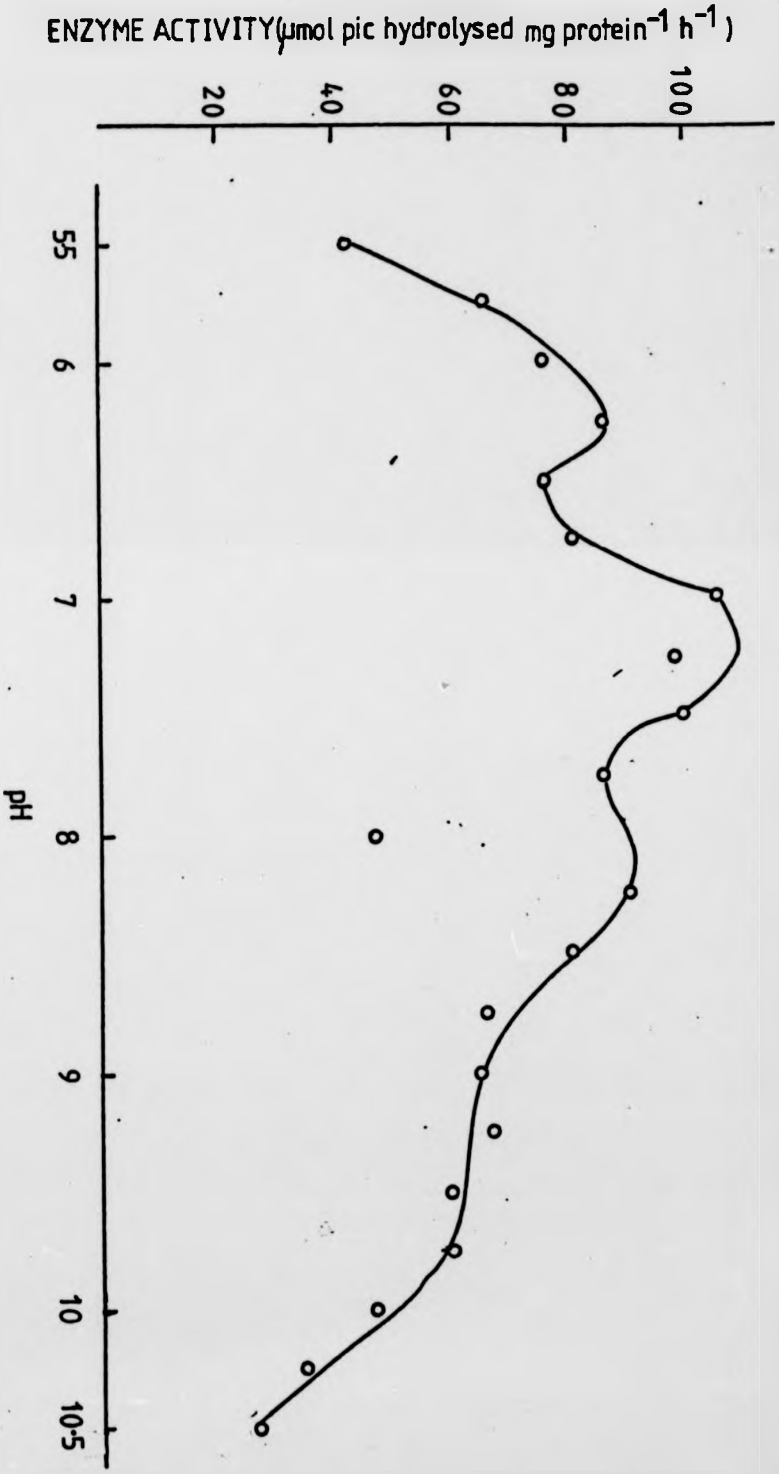

ENZYME ACTIVITY(μmol pic hydrolysed mg protein$^{-1}$ h$^{-1}$)
20
40
60
80
100
pH
5.5
6
7
8
9
10
10.5

Table 7.2 Inhbiition of picolinate 6-hydroxylase activity at pH 8.5 from mixed culture comprising A.faecalis and Alcaligenes sp2 growing at a dilution rate of $0.076h^{-1}$.

| Concn of inhibitor (mM) | Activity at 2.75mM picolinate | %age Activity of inhibited cultures |
|---|---|---|
| 0 | 92.6 | - |
| .302 | 90 | 97.3 |
| .906 | 82.1 | 88.7 |
| 1.509 | 79.7 | 86 |

### 7.1.3 Two Membered Mixed Culture Comprising A.faecalis and P.aeruginosa

The two membered mixed culture comprising A.faecalis and P.aeruginosa was studied in continous culture growing on picolinate and 36DCPA as the combined sources of carbon, at a concentration of 0.5 and 0.1g carbon $l^{-1}$ respectively in a "Quick fit" type fermenter (see section 2.3.2). The inoculum consisted of 100ml of late exponentially growing organisms of each species. A.faecalis was grown on picolinate but since some difficulty was experienced in growing P.aeruginosa in closed liquified culture on picolinate alone, a mixture of picolinate and glycerol (both at a concentration of 0.5g carbon $l^{-1}$) was used as carbon source for this organism.

The organisms were inoculated together and allowed to grow in closed culture for 48h before initiating the flow of fresh medium. 600ml of culture was harvested at all dilution rates studied and used to measure the parameters mentioned earlier.

The organisms were identified by colony morphology alone. A.faecalis exhibited large, opaque, cream-coloured colonies whilst P.aeruginosa showed large, greenish, slightly transluscent colonies.

Fig 7.10 shows the absorbance at 600nm and the dry weight of the culture at varying dilution rates. Both curves showed a peak at a dilution rate of $0.17h^{-1}$ and declined with increasing and decreasing dilution rates.

Figure 7.11 shows the activity of picolinate 6-hydroxylase at varying dilution rates. The activity showed a sharp peak to a maximum of 87 µmol picolinated hydrolysed (mg protein)$^{-1}$ $h^{-1}$ at a dilution rate of $0.2h^{-1}$. At higher dilution rates the activity decreased rapidly to 26.5 µmol picolinate hydroylsed at a dilution rate of $0.32h^{-1}$. It is interesting to note that during the period of this experiment there was no observed wall growth and this is reflected by no increase in the enzyme activity at very high dilution rates. At dilution rates below $0.2h^{-1}$ the activity decreased but appeared to reach a minimum level of approximately 45 µmol picolinate hydrolysed below a dilution rate of $0.05h^{-1}$.

Figure 7.10 Absorbance at 600nm and dry weight (mg $ml^{-1}$) of two species mixed culture containing A.faecalis and P.aeruginosa grown in chemostat culture with picolinate and 36DCPA as the source of carbons, at a concentration of 0.5 and 0.1 g carbon $l^{-1}$ respectively.


DRY WEIGHT (mg ml$^{-1}$)
·6
·4
·2

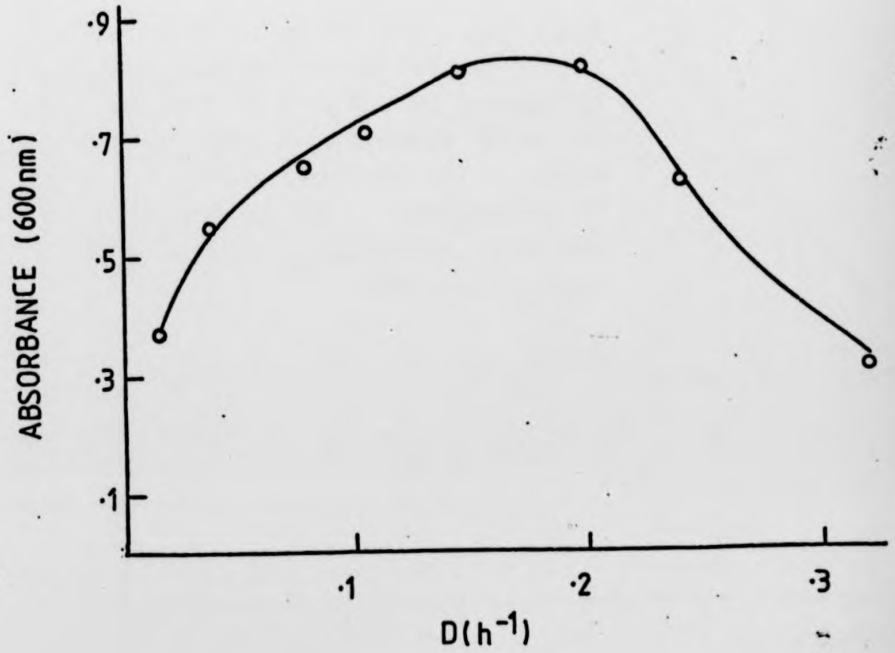

ABSORBANCE (600nm)
·9
·7
·5
·3
·1
·1
·2
·3
D(h$^{-1}$)

Figure 7.11 Picolinate 6-hydroxylase activity of two species mixed culture comprising A.faecalis and P.aeruginosa harvested from chemostat culture growing on picolinate and 36DCPA as the carbon source, at a concentration of 0.5 and 0.1 g carbon $l^{-1}$ respectively. (●) Activity at 0.558 mM picolinate, (o) maximum activity calculated from Lineweaver-Burk plots.

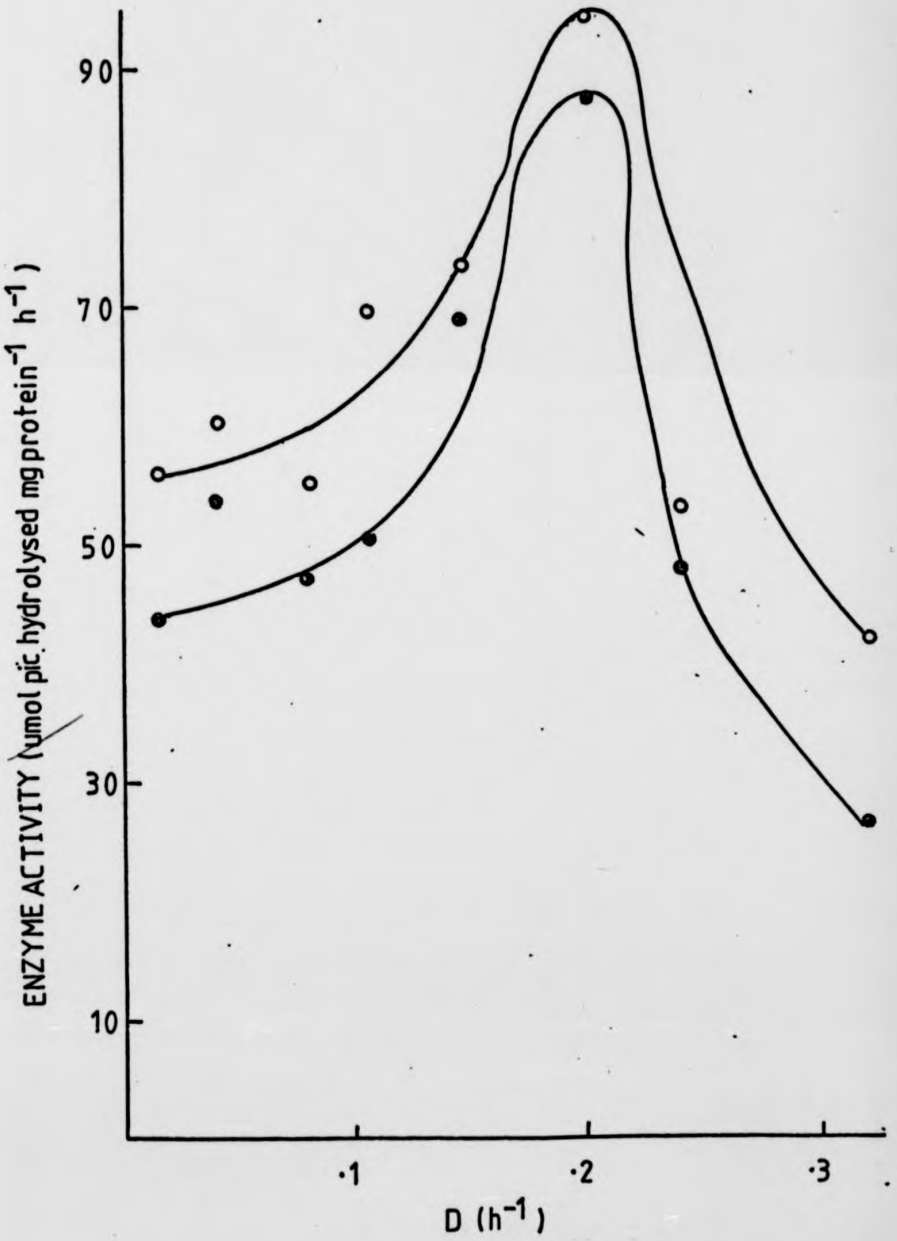

ENZYME ACTIVITY (umol pic. hydrolysed mg protein$^{-1}$ h$^{-1}$)
90
70
50
30
10
·1
·2
·3
D (h$^{-1}$)

Figure 7.12 shows the percentage of organisms present in the mixed culture A.faecalis was the predominant organism in the community, the percentage of this organism never falling below 90% at all the dilution rates studied. Initially the percentage of A.faecalis was 90% at a dilution rate increased, to a maximum of approximately 98% at a dilution rate of $0.1h^{-1}$ and fell slightly to 95% as the dilution rate increased to a maximum of $0.32h^{-1}$. The percentage of P.aeruginosa in the fermenter followed inversely the change in the A.faecalis population, initially having a high population, followed by a drop in numbers and a subsequent rise with increasing dilution rate.

It is possible that due to the limitations of serial dilutions and plating out that A.faecalis and P.aeruginosa were constant throughout the experiment at approximately 95% and 5% respectively.

At the optimum dilution rate for enzyme production of $D = 0.2h^{-1}$ the pH profile and inhibition of picolinate 6-hydroxylase by 36DCPA were studied. Figure 7.13 shows the pH profile of the enzyme (or enzymes) extracted from the mixed culture. Initially the activity was relatively low at 37 µmol picolinate hydrolysed $(mg\ protein)^{-1}\ h^{-1}$ but rose rapidly to a peak of 71 µmol picolinate hydrolysed at pH 6.25. After a small decrease in activity, with increasing pH, the activity rose again to a maximum of 85 µmol picolinate hydrolysed at pH 7.0 and exhibited a further peak of 68 µmol picolinate hydrolysed at pH 8.5. Thereafter, with increasing pH, the activity fell to a minimum of 21 µmol picolinate hydrolysed at pH 10.5.

Table 7.3 shows the activity of picolinate 6-hydroxylase during inhibition by 36DCPA at at a dilution rate of $0.2h^{-1}$. The apparent saturation constant (Km) and the apparent inhibition constant ($K_I$) were calculated by the methods described earlier. Km was found to be 0.77mM and $K_I$ was calculated to be 0.078mM, 0.08mM and 0.14mM from 0.302mM, 0.906mM and 1.509mM 36DCPA respectively. The average value of this constant was approximately 0.1mM.

Figure 7.12 species composition of two membered mixed culture comprising A.faecalis (o) and P.aeruginosa (•) in chemostat culture growing on picolinate and 36DCPA a as the source of carbon, at a concentration of 0.5 and 0.1 g carbon $l^{-1}$ respectively.

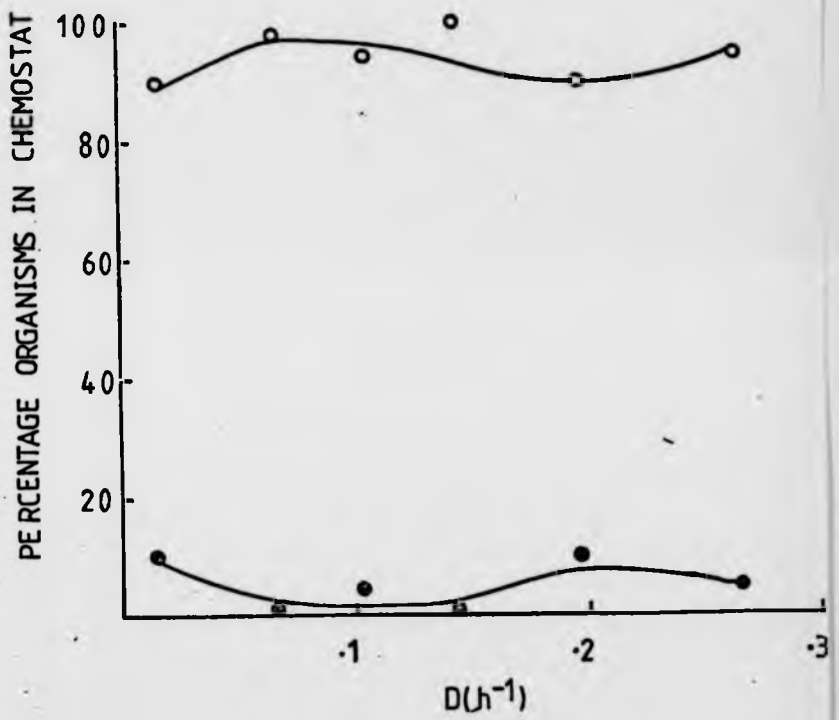

PERCENTAGE ORGANISMS IN CHEMOSTAT
100
80
60
40
20
·1
·2
·3
D(h⁻¹)

Figure 7.13 Picolinate 6-hydroxylase activity at varying pH from mixed culture comprising A.faecalis and P.aeruginosa. Extract was prepared from cells grown at the optimum dilution rate for enzyme production.

ty at
calis
cells
nzyme

### 7.1.4 Mixed Culture Comprising A.faecalis, B.licheniformis, Corynebacterium sp and Rhodococcus sp

The mixed culture comprising of the main primary utiliser, A.faecalis, and the three secondary utilisers was studied in continuous culture. The organisms were grown in a chemostat of the "Quick fit" type (see section 2.3.2). A.faecalis was grown on picolinate for the inoculum, B.licheniformis and Corynebacterium sp were grown on glucose and Rhodococcus sp was grown on glycerol. The organisms were simultaneously inoculated into the fermenter and allowed to grow in closed culture for 48h before initiating the flow of fresh medium. 600ml of culture was harvested at each steady state achieved and was used to measure the parameters mentioned earlier. The data for the percentage of organisms present in the mixed culture are not given since the three secondary utilisers were present at low levels (less than 1% at most times).

Figure 7.14 shows the biomass present in the fermenter, in terms of absorbance of 600nM and dry weight, at varying dilution rates. The absorbance exhibited a typical monoculture curve having a plateau of 0.7 absorbance units for most of the dilution rates studied but decreasing slightly at very low dilution rates and decreasing more markedly at high dilution rates.

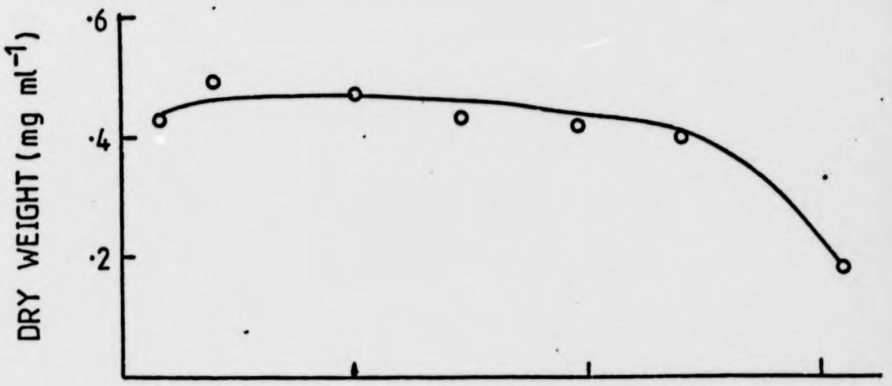
The dry weight of the culture followed a similar pattern but the plateau region decreased slightly with increasing dilution rate.

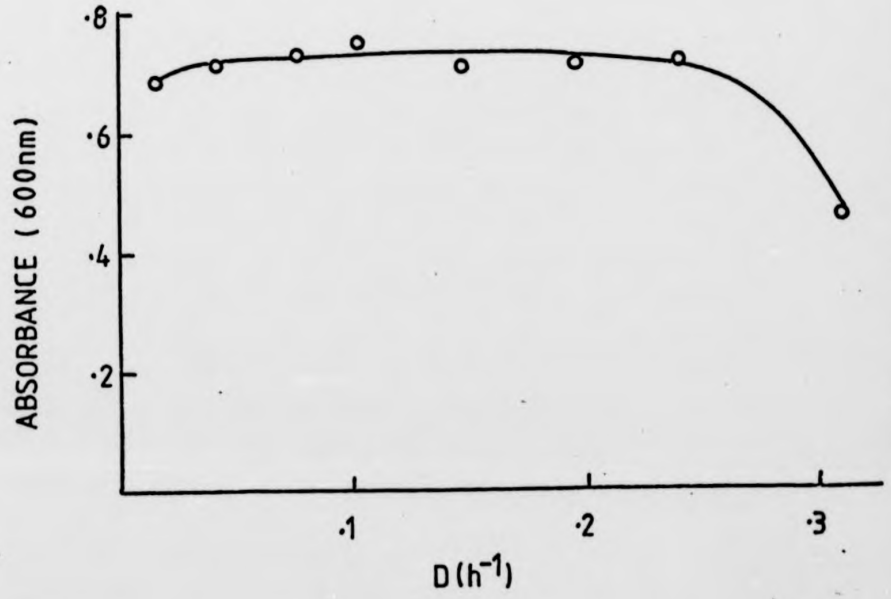
Figure 7.15 shows the activity of picolinate 6-hydroxylase in the four membered mixed culture at varying dilution rates. At the lowest dilution rate studied ($0.02h^{-1}$) the activity of the enzyme was approximately 63 µmol picolinate hydrolysed (mg protein)$^{-1}$ $h^{-1}$ and increased to a maximum of 79 µmol picolinate hydrolysed at a dilution rate of 0.075. Above this dilution rate the activity declined rapidly with increasing dilution rate to a value of 35 µmol picolinate hydrolysed at a dilution rate of $0.31h^{-1}$.

Table 7.3 Inhibition of picolinate 6-hydroxylase activity at pH 8.5 from two membered mixed culture of *A.faecalis* and *P.aeruginosa* growing at a dilution rate of $0.2h^{-1}$.

| Concn of inhibitor (mM) | Activity at 2.75mM picolinate | %age Activity of inhibited cultures |
|---|---|---|
| 0 | 69.1 | - |
| .302 | 68.4 | 99 |
| .906 | 65.2 | 94.5 |
| 1.509 | 63.9 | 92.5 |

Figure 7.14 Absorbance at 600nm and dry weight (mg $ml^{-1}$) of mixed culture comprising A.faecalis, B.licheniformis, Corynebacterium sp and Rhodococcus sp grown in chemostat culture with picolinate and 36DCPA as the carbon source at a concentration of 0.5 and 0.1 g carbon $l^{-1}$ respectively.


DRY WEIGHT (mg ml$^{-1}$)
·6
·4
·2


ABSORBANCE (600nm)
·8
·6
·4
·2
·1
·2
·3
D(h$^{-1}$)

At the optimum dilution rate for enzyme production ($0.08h^{-1}$) the pH profile and the inhibition of the extract from the mixed culture by 36DCPA was studied. Figure 7.16 shows the pH profile of the extract. The curve began at 46 µmol picolinate hydrolysed at pH 5.5, rose slowly to a maximum of 63 µmol picolinate hydrolysed at pH 7.25 and fell slowly to a minimum of 24 µmol picolinate hydrolysed at pH 10.5. This is another indication that multiple enzymes were present in the other mixed cultures because of the multiple peaks in the pH profiles of those cultures.

Table 7.4 shows the data on the inhibition of picolinate 6-hydroxylase by 36DCPA at a dilution rate of $0.08h^{-1}$. The apparent inhibition constant ($K_i$) from the concentration of 36DCPA used was calculated to be 0.22mm.

## 7.2 Discussion

Table 7.5 shows the data described in this chapter, in a more concise form, from the various types of mixed culture studied. By analysis of this table it should be possible to find indications of the parameters discussed in the beginning of the chapter.

The differences between the single substrate, picolinate and the mixed substrate, picolinate +36DCPA, on the growth of A. faecalis can be seen comparing columns A and B in the table. The maximum enzyme activity falls from 125 µmol picolinate hydrolysed to 115 µmol picolinate hydrolysed and the optimum dilution rate for enzyme production was decreased from 0.11 $h^{-1}$ to $0.08h^{-1}$ clearly indicating some inhibitory effect of the 36DCPA. However, because the organism could grow alone on the mixed substrate and remain in a stable state in the chemostat it may be considered that the organism played some part in the adaptation experiments discussed in chapter 5.

Figure 7.15 Picolinate 6-hydroxylase activity of mixed culture comprising A.faecalis, B.licheniformis, Corynebacterium sp., and Rhodococcus sp. grown in chemostat culture. (o) Activity at 0.558 mM picolinate (•) maximum activity calculated from Lineweaver-Burk plots.

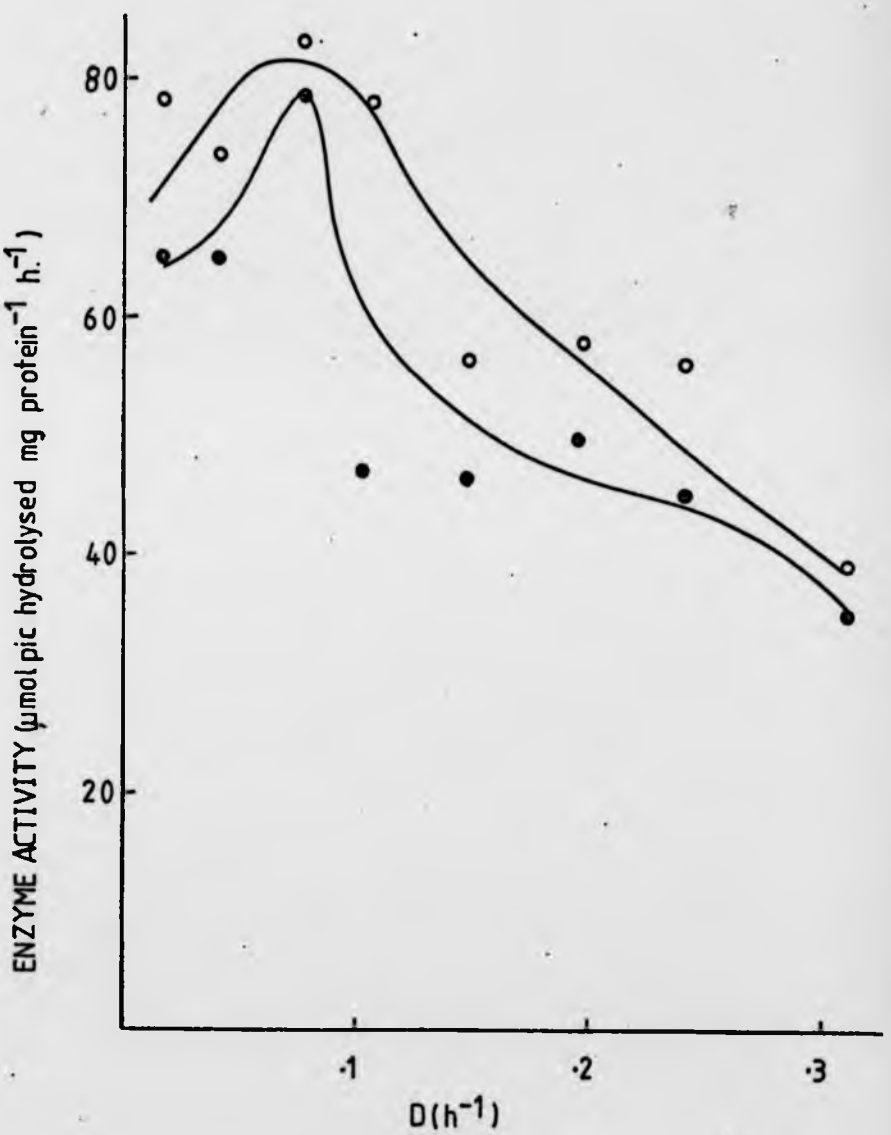

80
60
40
20
ENZYME ACTIVITY (µmol pic hydrolysed mg protein$^{-1}$ h.$^{-1}$)
·1
·2
·3
D(h$^{-1}$)

Figure 7.16 Picolinate 6-hydroxylase activity at varying pH from mixed culture comprising A.faecalis, B.licheniformis, Corynebacterium sp and Rhodococcus sp.. Extract was taken from culture growing at optimum dilution rate for enzyme production.

ENZYME ACTIVITY (μmol pic hydrolysed mg protein$^{-1}$ h$^{-1}$)
90
70
50
30
10
5·5
6
7
8
9
10
10·5
pH

Table 7.4 Inhibition of picolinate 6-hydroxylase activity at pH 8.5 from mixed culture comprising A.faecalis, B.lichenformis, Corynebacterium sp. and Rhodoccoccus sp. growing at a dilution rate of $0.08h^{-1}$

| Concn of inhibitor (mM) | Activity at 2.75mM picolinate | %age Activity of inhibited cultures |
|---|---|---|
| 0 | 83.2 | - |
| 0.302 | 78.6 | 94.5 |
| 0.906 | 70.2 | 84.5 |
| 1.509 | 49.2 | 59.2 |

Table 7.5 Summary of data calculated from the experiments described in section 7.1. (A) A.faecalis alone growing an picolinate alone. (B) A.faecalis growing on the mixed substrate. (C) A.faecalis and Alcaligenes sp2. (D) A.faecalis and P.aeruginosa. (E) A. faecalis and 3 secondary utilisers.

| PARAMETER | A | B | C | D | E |
|---|---|---|---|---|---|
| Maximum Enzyme Activity ($\mu$mol picolinate hydrolysed (mg protein)$^{-1}$ h$^{-1}$) | 125 | 115 | 87 | 87 | 79 |
| Optimum pH | - | 7.0/8.5 | 7.0 | 7.0/8.5 | 7.25 |
| $K_s$ (mM) | - | 0.526 | 0.065 | 0.77 | 0.714 |
| $K_I$* .302 mM | - | 0.0416 | 0.72 | 0.078 | 0.136 |
| .906 mM | - | 0.375 | - | 0.08 | 0.207 |
| 1.509 mM | - | 0.359 | 0.77 | 0.14 | 0.316 |
| Optimum D for Enzyme production | 0.11 | 0.08 | 0.075 | 0.2 | .075 |
| %age of other organisms (i. not A.faecalis) | - | - | 15% at D=02 dec to 0% at D=.2h-1 | 5% at all times | >1% for at all times & all organisms |

* Data calculated by computer analysis of Lineweaver-Burk plots.

The decrease in enzyme activity between the cultures B and C reflect the presence of Alcaligenes sp in the culture. The figures show a 25% decrease in the maximum enzyme activity. The reason for this may be due to Alcaligenes sp attacking picolinate in a different manner to that assayed for since this would effectively reduce the amount of picolinate present for attack by the A. faecalis hydroxylase. The decrease in maximum enzyme in the extract was reduced. The optimum dilution rate for maximum enzyme production remained at approximately $0.08h^{-1}$. This was indicative of Alcaligenes sp2 having little effect on the enzyme activity. Since this organism was only present, in measurable quantities, at low dilution rates it might have been expected that had the maximum amount of enzyme activity been related, in any way, to the organism, the optimum dilution rate would have altered to mirror this relation i.e. the optimum D would have decreased a little.

In culture D the maximum enzyme activity was again reduced, when compared with culture B, to 87 µmol picolinate hydrolysed and the same discussion may be applied.

However, the optimum dilution rate for enzyme production increased to $0.2h^{-1}$ and this can be seen to reflect the much faster growth rate of P.aeruginosa increasing the overall mixed culture growth rate and hence the growth rate of A. faecalis.

In culture E the activity of the enzyme was reduced further to 79 µmol picolinate hydrolysed and the optimum dilution rate for enzyme production was approximately equal to that of culture B and C ($0.075h^{-1}$). Similar discussions can be applied to culture E as those stated for culture C especially since it was not possible to demonstrate any picolinate activity in any of the secondary utilisers.

It should be noted at this point that the three mixed cultures studied were maintained stably during the time course of the fermenter studies. It is interesting to consider the reasons that enabled the initial community to exist if a more simple community (say for example culture C) could have performed an identical function. Why did the other organisms not wash out? An attempt to shed light on this problem was made during the study period using dialysis culture but due to technical problems and time limitations no results were found.

In culture E there were very few secondary utilisers and it may be that they produced some partly required nutrient. The reason for either, or both, of Alcaligenes sp2 and P.aeruginosa being present may have been to produce a nutrient or remove an inhibitor that increased the population of the secondary organisms to a more significant level in order that they might in turn produce a nutrient or remove an inhibitor.

The kinetic constants shown in Table 7.5 can also be used to give indications as to the interactions in the fermenter. In culture B it is clear that Ki calculated from 0.302mm inhibitor is in error and similarly in the table the other erroneous results can be observed. It would have been more complete to repeat these experiments but time limitations prevented this.

On comparing Ki for culture B and C it can be seen that a much higher inhibitor dissociation constant has been calculated. This in part reflects the observation made in section 5.2 that Alcaligenes sp2 was in some way responsible for the initial adaptation to tolerate 36DCPA.

In cultures D and E a higher substrate concentration is needed to achieve Vmax (the maximum enzyme activity) and this is presumably related to the presence of the other organisms.

The only work related to this is by Osman et al (1976). No reasons for the change in kinetic constants that were observed were given.

# CHAPTER 8

## CONCLUSIONS

The aim of this research was to study the breakdown, if any, of 36DCPA in the soil. It has clearly been established that 36DCPA is broken down in the soil by the mixed microbial community isolated. The half life of 36DCPA in various soils can be seen to be in the region of 70 days and, for an agricultural product that is applied annually, this is not a significant amount of time. On the evidence presented here, it is clear that accumulation of 36DCPA should not occur in the soil during the annual cycle.

From a microbiological viewpoint it is interesting to note that this is yet another stable mixed population that has been isolated, and partly characterised. The case for the prevalence of microbial communities in the natural environment has been added to. It would have been interesting, if time had permitted, to perform more continuous culture experiments on other combinations of the mixed culture and to perform the dialysis culture experiments mentioned in chapter 7 to try to establish the mechanism of the community structure.

The release of $^{14}CO_2$ from the soil degradation studies gave an indication that aerobic degradation of the herbicide was occurring and that further study of the degradation was feasible.

However, the enrichment process used in the soil columns did not produce any measurable degradation of 36DCPA. This may have been due to the recalcitrant nature of 36DCPA conferred on the molecule by the presence of the two chloride ions.

The picolinate community isolated remained stable over a long period of time and after adaptation to 36DCPA the stability was still maintained. However, as has been observed, (Cassel *et al*, 1966), the individual populations in the fermenter vessel fluctuate at constant dilution rates. It would be very interesting to study these fluctuating populations to try to establish the effect that an increase in one population had on any other population. It is also interesting to note that the rate of decrease in culture absorbance after the first and second additions of 36DCPA was almost equivalent to the rate of decrease that would be experienced with a culture washout. This may indicate that until day 100 the culture could not even tolerate the presence of 36DCPA in the medium.

The results given in Chapter 5 suggest that the mechanism of tolerance to 36DCPA, and possibly the mechanism of degradation is related to the two organisms Alcaligenes sp2 and the Corynebacterium sp. since the percentage of these organisms increased after the adaptation period. It would have been interesting to have studied these two organisms more closely both in pure culture and a mixed culture comprising the two organisms.

The hydroxylase studied shows the characteristics of a typical catabolic enzyme. At low dilution rates, and hence low growth rates, the enzyme activity is low due to low substrate concentrations. The activity increases as the dilution rate increases due to increased amount of substrate causing increased induction. Further increases of dilution rate, and hence substrate concentration lead to a decrease in enzyme concentration due to catabolic repression of enzyme synthesis.

The pH profiles of picolinate-6-hydroxylase from the various mixed populations studied (Figures 6.9, 7.9, 7.13 and 7.16) all show three peaks of activity at pH 6.25, 7 and 8.5. This may indicate that the organisms produce an acidic hydroxylase, a neutral hydroxylase and a basic hydroxylase.

This replication of the enzyme function is a potentially exciting topic for further study. It would be interesting to fractionate the cell extract and purify the enzyme to verify this replication.

One major disadvantage of studying the degradation of xenobiotic compounds is this manner is the dubious relation that any experiment can have the natural environment when the concentrations of compound added to the growing mixture are in excess of 1000 times the concentration used during field application.

Other problems with this work relate to the unrepeatability of the selection experiments since it may be that

(1) The same organisms are not present in the soil sample selected or

(2) A similar selection event may not occur because of a different mutation taking place.

CHAPTER 9

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APPENDIX 1

Program used to calculate linear regression by the Newton-Raphson method.

Workfile: SRALIB (08/15/78)

```
100         $RESET LINEINFO
200         $SET LIBRARY
300         $RESET NOBINDINFO
400         $RESET FREE
500               SUBROUTINE WLSSRA(X,Y,W,N,M,SM,C,SC)
600               REAL X(100), Y(100), W(100), SW, SWXY, SWX
700               REAL, SWY, SWXX, SWYY, DEL, M, C* SM, SC, Z, SY
800               INTEGER N,J
900               SW = 0.0
1000              SWXY = 0.0
1100              SWX = 0.0
1200              SWY =0.0
1300              SWXX = 0.0
1400              SWYY = 0.0
1500              DO 1 J = 1,N
1600              SW = SW + W(J)
1700              SWXY = SWXY + (W(J)*X(J)*Y(J))
1800              SWX = SWX + (W(J)*X(J))
1900              SWY = SWY + (W(J)*Y(J))
2000              SWXX = SWXX + (W(J)*X(J)*X(J))
2100        1     CONTINUE
2200              DEL = (SW*SWXX) - (SWX*SWX)
2300              M = ((SW*SWXY) - (SWX*SWY))/DEL
2400              C = ((SWY*SWXX) - (SWX*SWXY))/DEL
2500              DO 6 J-1, N
2600              Y(J) = (M*X(J) + C - Y(J))
2700              SWYY = SWYY + (Y(J)*Y(J)*W(J))
2800        6     CONTINUE
2900              SY = SQRT((SWYY)/(N - 2))
3000              SM = SY*SQRT(SW/DEL)
3100              SC = SY * SQRT(SWXX/DEL)
3200              WRITE(6,2)M
3300        2     FORMAT(/,10X,25HSLOPE =, E20.8)
3400              WRITE(6,4)SM
3500        4     FORMAT(/,10X,25HSTD. DEV. OF SLOPE =, E20.8)
3600              WRITE(6,3)C
3700        3     FORMAT(/,10X,25HINTERCEPT =, E20.8)
3800              WRITE(6,5)SC
3900        5     FORMAT(/,10X,25HST). DEV. OF INTERCEPT =, E20.8,/)
4000              RETURN
4100              END
```

G24 *The Growth of a Stable Mixed Culture on Picolinic Acid in Continuous-Flow Culture.*
D. Lovatt and J.H. Slater (Department of Environmental Sciences, University of Warwick, Coventry, CV4 7AL) and A.T. Bull (Department of Applied Biology, University of Wales Institute of Science and Technology, Cardiff CF1 3NU)

A stable mixed culture able to utilise pyridine-2-carboxylic acid (picolinic acid) as the sole carbon and energy source was isolated from soil by continuous-flow culture enrichment. The microbial community contained at least six different organisms, three of which were capable of growth on picolinic acid in pure culture. The three primary organisms were *Pseudomonas aeruginosa*, *Alcaligenes faecalis* and a second unidentified *Alcaligenes* species and the original culture was dominated by *Alcaligenes faecalis*. However, under constant dilution rate conditions there were considerable fluctuations in the percentage species composition of the community. The three secondary organisms, unable to grow on picolinic acid, were *Bacillus licheniformis*, a species of *Rhodococcus* and a *Corynebacterium* of the *aquaticum* type; these were presumed to be growing on either primary organism lysis products or excreted metabolites from the breakdown of picolinic acid. Cell-free extracts of the complete mixed culture converted picolinic acid to 6-hydroxypicolinic acid as measured by the change in absorbance at 300 nm (1). The hydroxylase had a pH optimum of 8.5 and maximum activity in cultures grown at $D = 0.16\ h^{-1}$.

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Initially the microbial community was unable to grow on 3,6-dichloropicolinic acid (36DCPA) in place of picolinic acid and the first attempts to adapt the microbial community to growth on a mixture of picolinic acid (0.85 g $l^{-1}$) and 36DCPA (0.27 g $l^{-1}$) resulted in culture washout. After three cycles of growth on picolinic acid alone followed by a period of growth on the mixed substrates, the mixed culture adapted to growth in the presence of 36DCPA. This was associated with a change in the percentage composition of the community since the unidentified *Alcaligenes* species became as abundant as *Alcaligenes faecalis*. There was some 36DCPA degradation, particularly at low growth rates where there was a 15% decrease in the 36DCPA concentration. However, the inability to detect free chloride ions in the culture suggested that 36DCPA was not completely degraded.

1. Dagley, S. and Johnson, P.A. (1963) *Biochimica et Biophysica Acta* 78, 577.

# The Growth of *Pseudomonas putida* on Chlorinated Aliphatic Acids and its Dehalogenase Activity

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Two strains of *Pseudomonas putida*, S3 and P3, were shown to contain dehalogenase activity against monochloroacetate, dichloroacetate, 2-monochloropropionate and 2,2′-dichloropropionate but differed markedly in their levels of enzyme activity. Strain S3 had activities of less than 1 $\mu$mol substrate converted (mg protein)$^{-1}$ h$^{-1}$ and was unable to grow on any of nine chlorinated compounds tested. Strain P3 had enzyme activities 10 to 40 times greater than those of strain S3 but was capable of growth only on 2-monochloropropionate and 2,2′-dichloropropionate. In strain P3, dehalogenase activity was induced by a number of chlorinated compounds other than those that acted as growth substrates. Strain P3 dehalogenase activity dehalogenated C-2 substituted compounds. The evidence of the dehalogenase activity profiles in chemostat cultures and from thermal denaturation experiments suggested that there was more than one dehalogenase enzyme in *P. putida* strain P3. In crude extract, the enzyme activity was optimal at pH 7·9 to 8·1 and apparent $K_m$ values were in the millimolar range for the four major substrates, monochloroacetate, dichloroacetate, 2-monochloropropionate and 2,2′-dichloropropionate.

## INTRODUCTION

Halogenated compounds occur widely in the biosphere either as natural products (Fowden, 1968; Suida & DeBernardis, 1973) or, increasingly, as xenobiotic compounds derived from the use of herbicides and pesticides (Kearney & Kaufman, 1969; Leasure, 1964; Audus, 1976). A few micro-organisms have been isolated that are capable of utilizing halogenated aliphatic acids as sole carbon and energy sources for growth. For example, Jensen (1957*a*, *b*, 1959, 1960) isolated various bacteria and fungi that were capable of growing on monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, 2-monochloropropionic acid and 2,2′-dichloropropionic acid. The active ingredient in the herbicide Dalapon, a widely and successfully used chlorinated aliphatic acid herbicide (Foy, 1969), is 2,2′-dichloropropionic acid, and this has been shown to be readily degraded by soil micro-organisms, particularly bacteria (Magee & Colmer, 1959; Hirsch & Alexander, 1960; Macgregor, 1963; Burge, 1969; Berry *et al.*, 1976).

Growth on these compounds depends on the induction of an enzyme known either as dehalogenase (Jensen, 1960, 1963) or as halidohydrolase (Goldman *et al.*, 1968; Goldman, 1972). Dehalogenases catalyse the hydrolytic removal of halogens, usually from C-2 substitutions, yielding either hydroxy- or oxo-carboxylic acids from the mono- or di-substituted acids, respectively (Davies & Evans, 1962; Kearney *et al.*, 1964; Goldman, 1965; Goldman *et al.*, 1968; Little & Williams, 1969, 1971).

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In this paper we describe a study of two [illegible] *Pseudomonas putida* strains (S3 and P3) (Senior *et al.*, 1976), differing in their capacity to utilize a series of chlorinated aliphatic propionic and butyric acids, and report the first characterization of their dehalogenase activities. Some of the results reported here have been the subject of a preliminary communication (Slater *et al.*, 1976).

## METHODS

*Abbreviations.* MCA, Monochloroacetic acid; DCA, dichloroacetic acid; TCA, trichloroacetic acid; 2MCPA, 2-monochloropropionic acid; 3MCPA, 3-monochloropropionic acid; 22DCPA, 2,2'-dichloropropionic acid; 23DCPA, 2,3-dichloropropionic acid; 2MCBA, 2-monochlorobutyric acid; 3MCBA, 3-monochlorobutyric acid; 4MCBA, 4-monochlorobutyric acid.

*Bacterial strains, maintenance and growth.* The two strains of *Pseudomonas putida*, S3 and P3, were isolated from a microbial community obtained by continuous-flow enrichment culture with 22DCPA as the sole carbon and energy source (Senior *et al.*, 1976).

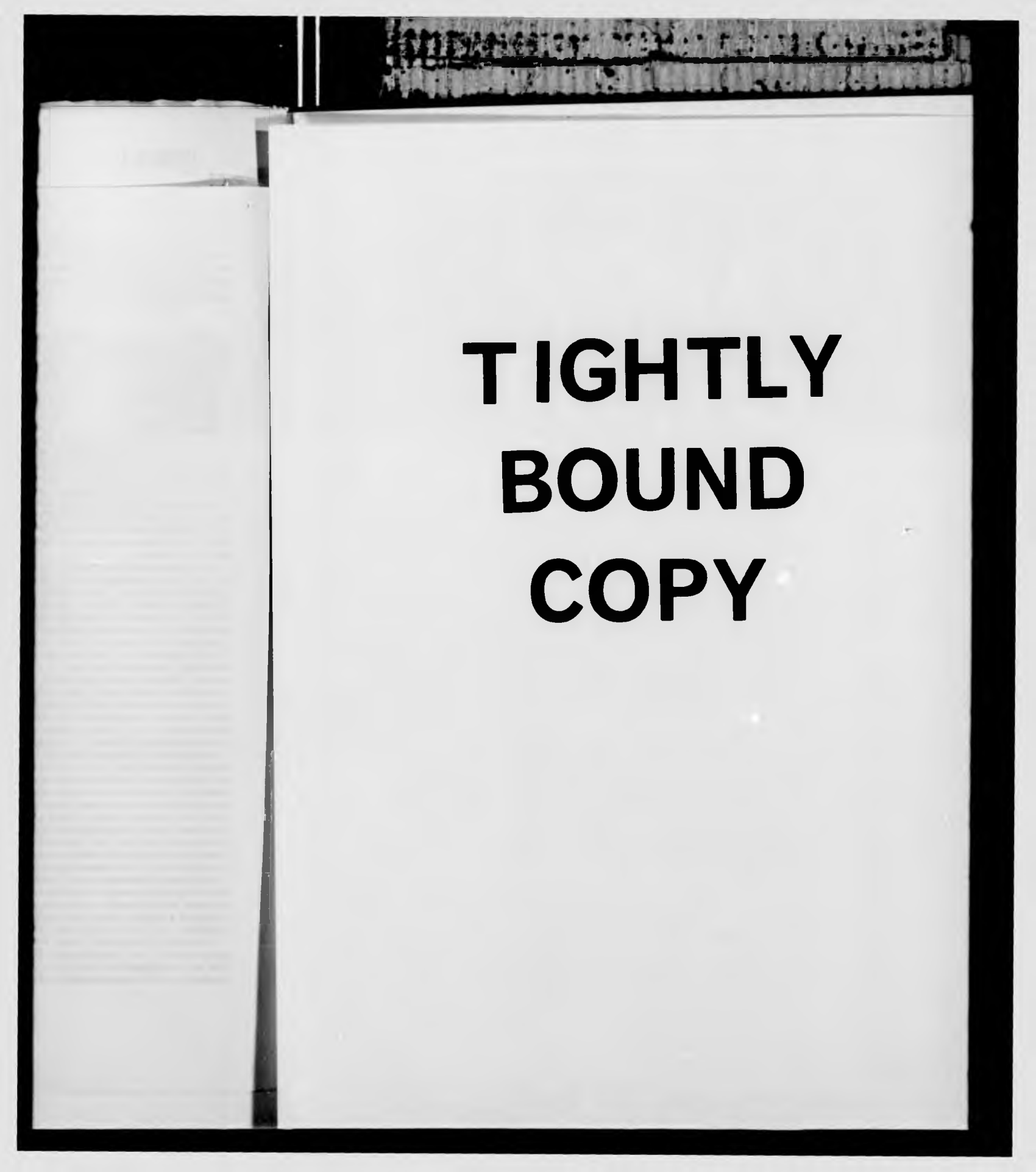
The growth medium contained (g $l^{-1}$): $K_2HPO_4$, 1·5; $KH_2PO_4$, 0·5; $(NH_4)_2SO_4$, 0·5; $MgSO_4.7H_2O$, 0·2; and 10·0 ml of a trace element solution containing (g $l^{-1}$): $Na_2EDTA.2H_2O$, 12·0; $FeSO_4.7H_2O$, 2·0; $CaCl_2$, 1·0; $Na_2SO_4$, 10·0; $ZnSO_4.7H_2O$, 0·4; $MnSO_4.4H_2O$, 0·4; $CuSO_4.5H_2O$, 0·1; $Na_2MoO_4.2H_2O$, 0·1; and 0·5 ml concentrated $H_2SO_4$. The pH of the mineral medium was adjusted to 7·0 before autoclaving. Carbon sources were supplied as different volumes of stock solutions (10%, w/v; pH 7·0) to give final concentrations of 0·5 g C $l^{-1}$. Solutions of chlorinated compounds were filter-sterilized to prevent thermal dechlorination. *Pseudomonas putida* strain S3 was maintained on mineral salts medium supplemented with sodium succinate and *P. putida* strain P3 with either 2MCPA or 22DCPA as the sole carbon and energy source. Closed cultures, usually 100 ml culture in 250 ml conical flasks, were incubated at 30 °C and growth was estimated by measuring the culture absorbance ($A_{600}$) in either a Corning model 252 colorimeter (red filter) or a Unicam SP1700 spectrophotometer.

Chemostat cultures were grown in LHE Series 500 Fermenter Units (LH Engineering, Stoke Poges, Bucks.) having a culture working volume of 0·8 l. Cultures were agitated at 1000 rev. $min^{-1}$ and supplied with air at the rate of 0·8 l $min^{-1}$. Fresh medium was fed to the growth vessel by a flow inducer (Watson Marlow, Falmouth, Cornwall). The growth temperature was controlled at 30 °C. The growth medium was that used for the closed cultures and gave carbon-limited growth. In some experiments two carbon sources (sodium succinate with a chlorinated aliphatic acid) were supplied, both at 0·5 g C $l^{-1}$.

*Estimation of chloride ions.* Chloride ions were determined in a Marius Chlor-0-Counter (F. T. Scientific, U.K.). Samples (0·1 or 1·0 ml) were added to 25 ml of a base counting solution (100 ml glacial acetic acid and 8·0 ml concentrated nitric acid in 1·0 l glass-distilled water) and 1·0 ml gelatin–thymol blue indicator solution (600 mg white powder gelatin, 10 mg thymol and 10 mg thymol blue pH indicator in 100 ml glass-distilled water). Free $Cl^-$ were titrated against $Ag^+$ generated coulometrically and this resulted in the precipitation of insolubie AgCl. The titration end-point was detected amperometrically by silver electrodes which measured the appearance of free $Ag^+$ and the titration time was directly proportional to the $Cl^-$ concentration. Up to 15·0 ml of samples could be assayed for each 25·0 ml base counting solution and the instrument detected 0·2 $\mu$mol $Cl^-$ per sample.

*Measurement of dehalogenase activity.* Enzyme activity, unless otherwise stated, was measured in crude extracts and, occasionally, in whole organism preparations. Cultures were harvested by centrifuging at 5000 *g* for 15 min, and the bacterial pellet was washed once and resuspended in ice-cold 0·02 M-$NaH_2PO_4/Na_2HPO_4$ buffer, pH 7·0. In the whole organism assay, bacterial membranes were disrupted by adding 0·1 ml toluene to 30 ml bacterial suspension and vigorously mixing for 30 s. For crude extracts, the organisms were disrupted by two passages through a French pressure cell at 83 MPa; the remaining whole organisms and cell debris were removed by centrifuging at 30000 *g* for 45 min. Dehalogenase activity was assayed in a reaction mixture containing, in 7·5 ml: 500 $\mu$mol $NaH_2PO_4/Na_2HPO_4$ buffer pH 7·9; 5 $\mu$mol NaCl; 100 to 200 $\mu$mol chlorinated aliphatic acids (solutions adjusted to pH 7·0), to initiate the reaction; and up to 2·0 ml crude extract (or up to 2·0 ml toluene-treated bacterial suspension) containing a maximum of 5·0 mg protein. The incubation temperature was 30 °C and 1·0 ml samples were removed for $Cl^-$ estimation at intervals over the first 45 min of reaction. Protein was estimated by the Lowry method with bovine serum albumin as the standard. Enzyme specific activities are given as $\mu$mol substrate converted (mg protein)$^{-1}$ $h^{-1}$. Enzyme activity as a function of assay pH was determined using the following buffers: pH 5·7 to 7·9, $NaH_2PO_4/Na_2HPO_4$; pH 7·6 to 8·6, Tris/maleate; pH 8·6 to 10·0, glycine/NaOH.

*Expression of dehalogenase activity.* For the determination of dehalogenase activity in non-growing *P. putida* strain S3, 1·5 l succinate-grown, late-exponential phase organisms were harvested at 5000 *g* for 15 min and resuspended in 300 ml 0·2 M-$NaH_2PO_4/Na_2HPO_4$ buffer, pH 7·0. Aliquots (3 × 100 ml) were incubated at 30 °C with gentle shaking. No additions were made to one of the flasks but to the second and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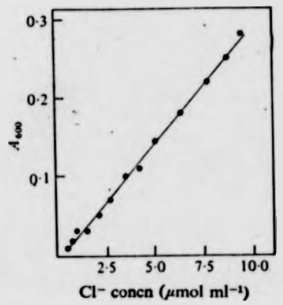


Fig. 1. Differential plot of the increase in culture absorbance against the increase in $Cl^-$ concentration for *Pseudomonas putida* strain P3 grown on 2MCPA in closed culture.

third, 2MCPA and 22DCPA were added, respectively, to give final concentrations of 0·5 g $Cl^{-1}$. After 24 h incubation under these starvation conditions the organisms were harvested and assayed for dehalogenase activity.

For experiments with growing cultures of *P. putida* strain P3, chlorinated aliphatic acids were added to mid-exponential phase cultures to give final concentrations of 0·5 g C $l^{-1}$. These cultures were grown in the presence of 0·5 μmol NaCl $ml^{-1}$ to ensure a low background level of $Cl^-$ that facilitated accurate $Cl^-$ estimation. In some experiments, the appearance of dehalogenase activity was measured in terms of dehalogenation of the inducer compound only, while in others, organisms were harvested and assayed for dehalogenase activity against a range of substrates.

*Thermal stability of dehalogenase activity.* Crude extracts were incubated at 50 °C, and samples were taken at intervals over 50 min incubation, cooled quickly in ice and immediately assayed for MCA, DCA, 2MCPA and 22DCPA dehalogenase activity. The changes in activity with time at 50 °C were expressed as percentages of the initial activity for each substrate.

*Materials.* All chemicals were of the highest purity commercially available. MCA was obtained from May & Baker, Dagenham; DCA and 2MCPA from Koch-Light; TCA from Sigma; 23DCPA and 2MCBA from Fluka (supplied by Fluorochem, Glossop, Dorset); 3MCBA from Pfaltz & Bauer, Flushing, N.Y., U.S.A.; 3MCPA from Hopkin & Williams, Chadwell Heath, Essex; and 4MCBA from R. N. Emanuel, Wembley, London. 22DCPA was purified by fractional distillation from technical grade material provided by the Dow Chemical Co., Kings Lynn, Norfolk.

## RESULTS

### *Growth characteristics of Pseudomonas putida strains* S3 *and* P3

The two strains of *P. putida* had similar specific growth rates ($\mu$) when the carbon and energy sources were propionate ($\mu = 0{\cdot}30\ h^{-1}$), lactate ($\mu = 0{\cdot}41\ h^{-1}$), pyruvate ($\mu = 0{\cdot}41\ h^{-1}$) and succinate ($\mu = 0{\cdot}44\ h^{-1}$). They differed in their capacity to grow on chlorinated propionates: *P. putida* strain P3 grew on 2MCPA ($\mu = 0{\cdot}30\ h^{-1}$) and 22DCPA ($\mu = 0{\cdot}12\ h^{-1}$), whereas *P. putida* strain S3 was unable to grow on 2MCPA or 22DCPA in liquid culture. *Pseudomonas putida* strain S3, however, did produce very small colonies after 48 to 72 h on solidified medium when 2MCPA was the carbon source. The growth of *P. putida* strain P3 on 2MCPA and 22DCPA was directly correlated with the dechlorination of the carbon sources since differential plots of culture absorbance against $Cl^-$ release were linear (Fig. 1).

*Pseudomonas putida* strain P3 did not show diauxic growth in medium containing any combination of unchlorinated carbon source with 2MCPA or 22DCPA, both substrates being used simultaneously. The addition of either 2MCPA or 22DCPA to exponentially growing cultures of *P. putida* strain P3 caused only minor perturbations in growth (Fig. 2*a*) and resulted in the appearance of $Cl^-$, indicative of the metabolism of the chlorinated aliphatic acids, after a lag period varying between 60 and 90 min (Fig. 2*b*). The simultaneous

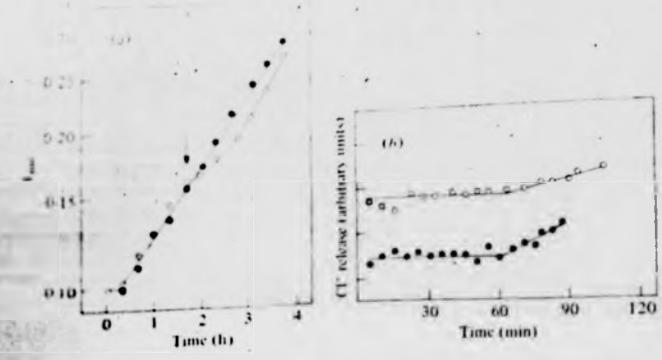


**Fig. 2.** Effect of addition of 2MCPA and 22DCPA to cultures of *Pseudomonas putida* strain P3 growing on succinate. (*a*) Effect on growth; arrow indicates time of addition of either 2MCPA (○) or 22DCPA (●) to give final concentrations of 0·5 g C $l^{-1}$. (*b*) Release of $Cl^-$ from 2MCPA (○) and 22DCPA (●) added at time 0 (arrowed in *a*).

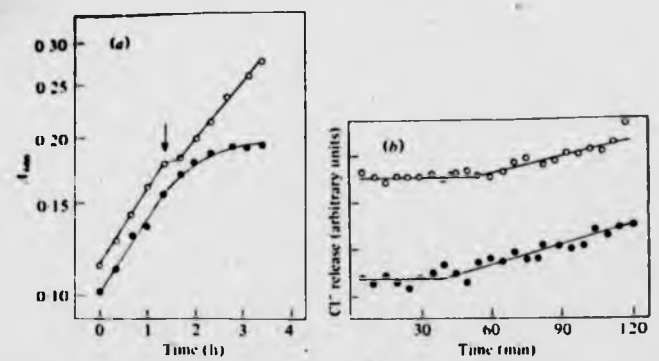


**Fig. 3.** Effect of addition of MCA and DCA to cultures of *Pseudomonas putida* strain P3 growing on succinate. (*a*) Effect on growth; arrow indicates time of addition of either MCA (○) or DCA (●) to give final concentrations of 0·5 g C $l^{-1}$. (*b*) Release of $Cl^-$ from MCA (○) and DCA (●) added at time 0 (arrowed in *a*).

addition of chloramphenicol (final concentration 2·0 $\mu$g $ml^{-1}$) completely prevented $Cl^-$ release. The addition of 2MCPA or 22DCPA to exponentially growing cultures of *P. putida* strain S3 did not influence the growth nor did it induce any significant $Cl^-$ release.

*Pseudomonas putida* strain P3 could not utilize either MCA or DCA as the sole carbon source although addition of these substrates to cultures growing exponentially on succinate resulted in their dehalogenation, $Cl^-$ release occurring after lag periods varying between 40 and 60 min (Fig. 3*b*). The addition of both MCA and DCA affected growth: DCA caused growth to cease 90 min after its addition but growth resumed once dehalogenation was complete (Fig. 3*a*). In contrast *P. putida* strain S3 was unable to dechlorinate added MCA or DCA.

Neither strain was able to use TCA, 3MCPA, 23DCPA, 2MCBA or 3MCBA as carbon and energy sources for growth.

Table 1. *Dehalogenase activity against a range of chlorinated aliphatic acids in Pseudomonas putida strain* P3 *grown on 2MCPA and in P. putida strain* S3 *starved for 24 h in the presence of 2MCPA*

Enzyme activities were calculated on the basis of μmol substrate converted (mg protein)$^{-1}$ h$^{-1}$ and relative activities are expressed as a percentage of the activity against 2MCPA.

| | Relative enzyme activity | | |
|---|---|---|---|
| | Strain P3 | | Strain S3 |
| Dehalogenase substrate | Fresh crude extract | Crude extract stored for 24 h at 4 °C | Fresh whole organisms |
| MCA | 168 | 123 | ND |
| DCA | 90 | 66 | 244 |
| TCA | 13 | 16 | ND |
| 2MCPA | 100 | 100 | 100 |
| 3MCPA | 3 | 8 | ND |
| 22DCPA | 48 | 43 | 58 |
| 23DCPA | 18 | ND | ND |
| 2MCBA | 17 | 50 | ND |
| 3MCBA | 0 | 0 | ND |
| 4MCBA | 0 | 0 | ND |

ND, Not determined.

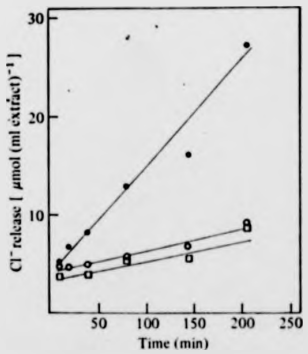


Fig. 4. Dehalogenase activity in *Pseudomonas putida* strain S3 originally grown on succinate and subsequently starved for 24 h in the presence of 2MCPA. Activity was measured against DCA (●), 2MCPA (○) and 22DCPA (□).

### *Dehalogenase activity in Pseudomonas putida strains* S3 *and* P3

*Enzyme activity and specificity.* Extracts of *P. putida* strain P3 grown on 2MCPA or 22DCPA contained dehalogenase activity whereas no activity could be detected in organisms grown on succinate, pyruvate, lactate or propionate. Table 1 shows that the major activities were for MCA, DCA, 2MCPA and 22DCPA. In addition, there was significant dehalogenation activity against a number of other substrates substituted on the C-2 position, particularly 2MCBA, and extremely low activity against one C-3 substituted compound, 3MCPA. There was considerable variation in the ratios of the relative enzyme activities in different crude preparations, the one exception was the 2MCPA:22DCPA ratio which

Table 2. *Apparent Michaelis–Menten ($K_m$) constants for dehalogenase activity in crude extracts of Pseudomonas putida strain P3 grown on 2MCPA in closed culture*

| Expt | Apparent $K_m$ (mM) | | | |
|---|---|---|---|---|
| | MCA | DCA | 2MCPA | 22DCPA |
| 1 | 8·0 | 0·99 | 2·9 | 1·2 |
| 2 | ND | 0·95 | 3·1 | 2·6 |

ND, Not determined.

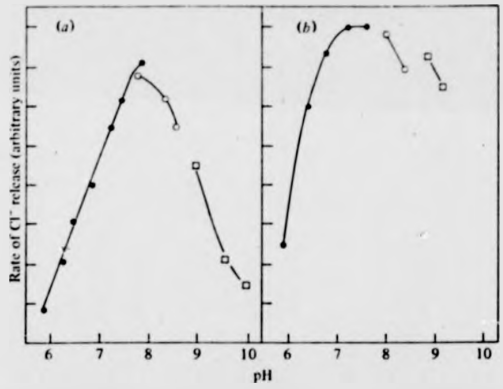


Fig. 5. Effect of pH on dehalogenase activity against DCA (*a*) and 2MCPA (*b*). Buffers used were $NaH_2PO_4/Na_2HPO_4$ (●), Tris/maleate (○) and glycine/NaOH (□).

remained constant at 2:1. Furthermore, marked changes in the various dehalogenase activities occurred in extracts stored at 4 °C for varying periods of time, there being a particularly rapid loss of DCA dehalogenation capability (Table 1). These variations in the enzyme specific activities are discussed further in relation to chemostat experiments.

Dehalogenase activity in *I. putida* strain S3 was more difficult to demonstrate because this organism could not be grown on chlorinated propionates or acetates. However, specific enzyme activities against DCA, 2MCPA and 22DCPA, at least an order of magnitude lower than those recorded in strain P3, were detected in organisms first grown on succinate and subsequently starved for 24 h in the presence of 2MCPA (Table 1, Fig. 4). No dehalogenase activity was detected in bacterial suspensions starved in the absence of a chlorinated compound or in the presence of 22DCPA. Activity could not be detected against the other potential substrates.

Apparent Michaelis–Menten constants for the four major substrates were determined for crude extracts of 2MCPA-grown *P. putida* strain P3 from Lineweaver–Burk plots (Table 2). There was insufficient activity to determine the apparent $K_m$ values for the dehalogenase activity in *P. putida* strain S3.

*pH optima.* The dehalogenation activity for the four major substrates had two markedly different pH profiles. The pH optima for MCA and DCA dehalogenation lay between pH 7·9 and 8·1 and the activity declined rapidly on either side of this optimum (Fig. 5*a*). The pH–activity profiles for 2MCPA and 22DCPA, however, had considerably broader optima with a mid-point at approximately pH 8·0 (Fig. 5*b*).

*Dehalogenase activity in chemostat cultures.* In view of the considerable variations in enzyme specific activity in closed cultures, the influence of growth conditions and growth

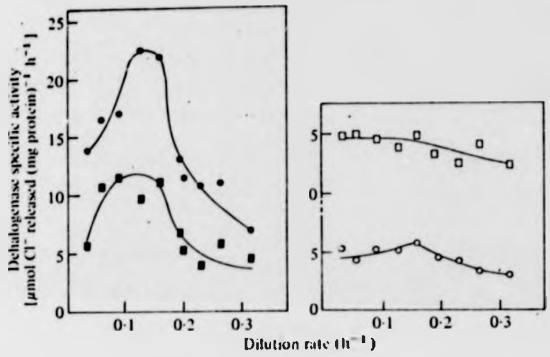


**Fig. 6.** Influence of dilution rate on the dehalogenase specific activity of *Pseudomonas putida* strain P3 grown under 2MCPA-limited conditions. Dehalogenase activity was measured against DCA (●), MCA (■), 2MCPA (○) and 22DCPA (□).

**Table 3.** *Dehalogenase activity against a range of chlorinated aliphatic acids in Pseudomonas putida strains* P3 *and* S3 *grown in chemostat culture*

Dehalogenase specific activities are expressed as μmol substrate converted (mg protein)$^{-1}$ h$^{-1}$. Relative enzyme activities are expressed as a percentage of the activity against 2MCPA. The dehalogenase activities for *P. putida* strain S3 were all measured at $D = 0{\cdot}10\ h^{-1}$; no attempt was made to determine $D$ for maximum activity.

| | Growth conditions | | | Dehalogenase | | |
|---|---|---|---|---|---|---|
| Strain | Growth-limiting substrate | Enzyme inducer | $D$ for maximum activity ($h^{-1}$) | Substrate | Maximum specific activity | Relative enzyme activity |
| P3 | 2MCPA | 2MCPA | 0·13 | MCA | 12·0 | 226 |
| | | | 0·13 | DCA | 11·2 | 211 |
| | | | 0·04–0·15 | 2MCPA | 5·3 | 100 |
| | | | 0·04–0·15 | 22DCPA | 2·5 | 47 |
| P3 | Succinate | DCA | 0·10 | MCA | 29·3 | 172 |
| | | | 0·08 | DCA | 38·5 | 226 |
| | | | 0·10 | 2MCPA | 17·0 | 100 |
| | | | 0·10 | 22DCPA | 7·9 | 46 |
| S3 | Succinate | 2MCPA | ND | MCA | 0·9 | 113 |
| | | | ND | DCA | 0·6 | 75 |
| | | | ND | 2MCPA | 0·8 | 100 |
| | | | ND | 22DCPA | 0·3 | 38 |

ND, Not determined.

rate on dehalogenase activity was examined for both strains in chemostat culture. Two different patterns of dehalogenase specific activity as a function of dilution rate were observed in steady-state cultures of *P. putida* strain P3 growing on 2MCPA as the limiting substrate (Fig. 6). The 2MCPA and 22DCPA dehalogenase specific activity changes were similar, each showing a slight increase with decreasing dilution rate and an indication of a small peak of activity at a dilution rate of approximately $D = 0{\cdot}15\ h^{-1}$. With MCA and DCA as the substrates, however, a pattern of changing enzyme specific activity characteristic of catabolic enzymes (Clarke & Lilly, 1969; Dean, 1972) was observed. There were significant activity maxima at $D = 0{\cdot}13\ h^{-1}$; with increasing dilution rate activity declined,

Table 4. *Induction of dehalogenase activity in Pseudomonas putida strain P3 by a range of chlorinated aliphatic acids*

Dehalogenase specific activities are expressed as μmol substrate converted (mg protein)$^{-1}$ h$^{-1}$. Values in parentheses indicate the activities as a percentage of those against 2MCPA.

| Inducer | Dehalogenase activity | | | |
|---|---|---|---|---|
| | MCA | DCA | 2MCPA | 22DCPA |
| MCA | 11·9 (290) | 9·0 (220) | 4·1 (100) | 1·9 (46) |
| DCA | 5·8 (290) | 2·2 (110) | 2·0 (100) | 1·1 (55) |
| TCA | 14·9 (257) | 18·8 (324) | 5·8 (100) | 3·4 (58) |
| 2MCPA | 15·4 (223) | 21·2 (307) | 6·9 (100) | 3·7 (54) |
| 3MCPA | 6·7 (372) | 12·2 (678) | 1·8 (100) | 1·3 (72) |
| 22DCPA | 19·9 (243) | 21·7 (265) | 8·2 (100) | 5·4 (65) |
| 2MCBA | 15·5 (267) | 15·3 (264) | 5·8 (100) | 3·4 (59) |
| 3MCBA | 5·2 (273) | 5·0 (263) | 1·9 (100) | 1·4 (74) |
| 4MCBA | 3·0 (300) | 5·7 (570) | 1·0 (100) | 0·7 (70) |

presumably as a result of catabolite repression, while activity also declined with decreasing dilution rate due to the gradual lowering of the residual growth-limiting substrate/inducer concentration. These patterns of changes in enzyme activity with dilution rate are consistent with the considerable variations in the relative enzyme activities observed in closed culture. The maximum enzyme activities observed in chemostat culture (Table 3) showed a constant ratio of activities for 2MCPA:22DCPA, which was also similar to the ratios found in closed culture (Table 1). However, the MCA:2MCPA and DCA:2MCPA activity ratios were variable and significantly different from the average ratios shown in closed culture, particularly the latter ratio which indicated a threefold increase in the DCA activity.

*Pseudomonas putida* strain P3 was also grown on succinate in the presence of DCA by first establishing a steady-state chemostat culture growing on succinate alone and subsequently switching the medium supply to the mixed substrates. As a result of the initial gradual addition of DCA to the growing culture, adequate dehalogenation activity was induced to ensure the complete degradation of the supplied DCA and thereby prevent the accumulation of growth inhibitory concentrations of undegraded DCA. In these cultures, the MCA and DCA dehalogenase activity profiles, as a function of dilution rate, were similar to those of 2MCPA-limited cultures, although the peaks of activity occurred at lower dilution rates (Table 3). Furthermore, in the chemostat cultures grown on succinate with DCA, the maximum enzyme specific activities were considerably higher, the DCA dehalogenase activity being 3·5 times greater than in 2MCPA-limited chemostat cultures. Similarly, 2MCPA and 22DCPA dehalogenase activities were approximately three times higher in the presence of DCA and showed obvious peaks of activity at a common dilution rate of $D = 0{\cdot}10\ h^{-1}$. With the exception of the MCA dehalogenase activity, the relative enzyme activities were similar in DCA- and 2MCPA-induced cultures and there was a constant 2MCPA:22DCPA ratio of about 2:1 in all steady-state cultures examined.

Organisms of *P. putida* strain S3 synthesized low levels of dehalogenase activity against the four major substrates when exposed to 2MCPA in chemostat cultures growing on succinate at a single dilution rate of $0{\cdot}10\ h^{-1}$ (Table 3). The specific activities reported are the average of five separate determinations; no attempt was made to measure the enzyme activities as a function of growth rate. These results are consistent with the inducibility of dehalogenase in non-growing organisms of strain S3 by 2MCPA reported above.

*Induction of dehalogenase activity by chlorinated aliphatic acids.* The capacity of metabolizable and non-metabolizable chlorinated aliphatic acids to induce dehalogenase activity against MCA, DCA, 2MCPA and 22DCPA in *P. putida* strain P3 was examined in closed cultures growing on succinate (Table 4). All the compounds examined were able to induce dehalogenase activity against all four enzyme substrates, although some, including MCA

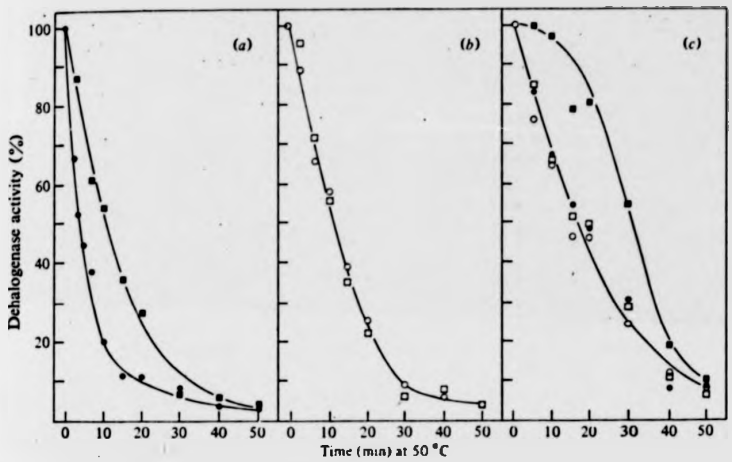


Fig. 7. Stability of dehalogenase activities during incubation at 50 °C of freshly prepared crude extracts (*a*, *b*) and crude extracts stored at 4 °C for 48 h (*c*) of *Pseudomonas putida* strain P3 grown on 2MCPA in closed culture. The activities against DCA (●), MCA (■), 2MCPA (○) and 22DCPA (□) are expressed as a percentage of the maximum activity for each substrate at time 0.

Table 5. *Relative dehalogenase activities in freshly prepared extracts of Pseudomonas putida strain* P3 *after incubation at 50 °C for various times*

The DCA dehalogenase activity was standardized to 1·0 at each incubation time and all other activities are expressed relative to this.

| Incubation time at 50 °C (min) | Relative dehalogenase activity | | | |
|---|---|---|---|---|
| | MCA | DCA | 2MCPA | 22DCPA |
| 0 | 1·0 | 1·0 | 0·8 | 0·4 |
| 3 | 1·7 | 1·0 | 1·4 | 0·7 |
| 7 | 1·5 | 1·0 | 1·2 | 0·7 |
| 10 | 3·4 | 1·0 | 2·8 | 1·3 |
| 15 | 6·7 | 1·0 | 5·6 | 2·4 |
| 20 | 7·6 | 1·0 | 5·4 | 2·3 |
| 30 | 4·6 | 1·0 | 2·4 | 1·8 |

TCA, 2MCPA, 22DCPA and 2MCBA, were better inducers than others. There were considerable variations in the absolute enzyme specific activities and variations in the ratios of activities against MCA:DCA:2MCPA; for example, the DCA:2MCPA activity ratio varied from 1·1:1·0 to 6·8:1·0. However, with the exception of the induction by 3MCPA, MCBA and 4MCBA, the ratios of activities against 2MCPA:22DCPA were constant and in line with the growth experiments.

*Thermal stability of dehalogenase activity.* The variation in dehalogenase activities in the growth and induction experiments suggested that *P. putida* strain P3 contained more than one dehalogenase protein and that each catalysed dechlorination of one or more different substrates. Further evidence for this possibilty was obtained from thermal denaturation experiments. The denaturation curves for freshly prepared extracts of *P. putida* strain P3 showed marked differences depending on the substrate (Fig. 7*a*, *b*). Thus

Table 6. [illegible]

The DCA dehalogenase activity was standardized to 1·0 at each incubation time and all other activities are expressed relative to this.

| Incubation time at 50 °C (min) | Relative dehalogenase activity | | | |
|---|---|---|---|---|
| | MCA | DCA | 2MCPA | 22DCPA |
| 0 | 1·6 | 1·0 | 2·1 | 1·6 |
| 5 | 2·1 | 1·0 | 2·1 | 1·5 |
| 10 | 2·6 | 1·0 | 2·1 | 1·6 |
| 15 | 2·6 | 1·0 | 2·0 | 1·4 |
| 20 | 3·0 | 1·0 | 2·1 | 1·6 |
| 30 | 3·3 | 1·0 | 2·0 | 1·3 |

the original DCA dehalogenase activity was lost after 10 min incubation at 50 °C whereas the MCA, 2MCPA and 22DCPA dehalogenase activities were reduced by only 40% during the same period. A comparison of the activity ratios showed that the loss of DCA dehalogenase activity was not correlated with the loss of MCA, 2MCPA and 22DCPA dehalogenase activity (Table 5). There was a parallel loss of 2MCPA and 22DCPA dehalogenase activities which also seemed to be related to the loss of MCA activity. We had observed previously (see above) that much of the DCA dehalogenase activity in crude extracts was lost after storage at 4 °C. A comparison of the thermal denaturation curves for stored extracts showed a strikingly parallel loss of activity for DCA, 2MCPA and 22DCPA (Fig. 7*c*, Table 6). In these extracts the MCA dehalogenase activity increased relative to standard DCA, 2MCPA or 22DCPA activities with an increasing period of denaturation. These results suggested that perhaps the MCA dehalogenase activity was not correlated with the other three activities.

## DISCUSSION

*Pseudomonas putida* strain P3 was isolated from a stable mixed culture growing on 22DCPA and which initially contained *P. putida* S3 (Senior *et al.*, 1976). It was suggested that strain P3 was a mutant of strain S3 differing in its capacity to metabolize chlorinated aliphatic acids and its dehalogenase activity. Mono-culture chemostat experiments (Senior *et al.*, 1976) and conventional selection experiments with and without mutagenesis on solid medium (A. J. Weightman & J. H. Slater, unpublished) have shown that it is possible to select for other strains from S3 that are phenotypically similar to P3. The results reported here show that the capacity of strain P3 to grow on chlorinated propionic acid was due to considerably elevated dehalogenase activities compared with strain S3, i.e. activities that were able to sustain the observed growth rates. The starvation experiments suggested that strain S3 was unable to use 22DCPA as a carbon source because the substrate did not induce dehalogenase activity, whereas the low enzyme activities induced by 2MCPA may have been high enough to account for its slow growth on this substrate. The inability of *P. putida* strain P3 to utilize MCA or DCA as carbon and energy sources, despite high dehalogenase activities towards these compounds, remains obscure and is currently being examined.

The capacity of the mutant *P. putida* strain P3 to grow on chlorinated aliphatic acids was the result of a general increase in a number of dehalogenase activities rather than a specific alteration towards a 22DCPA-specific dehalogenase and possibly reflected an alteration in the organism's regulatory mechanism. Strain P3 was not a constitutive mutant as may have been expected as a result of selection in continuous-flow culture (Hartley *et al.*, 1972). The dehalogenase activities were induced despite the presence of alternative, readily metabolizable carbon sources, a property that may be significant since the strain was selected

an environment that contained other carbon sources. Moreover, all the dehalogenase tivities were induced by a range of metabolizable and non-metabolizable chlorinated phatic acids, some of which were not involved in the original selection of strain P3.

Dehalogenase activity was mainly against C-2 substituted aliphatic acids, an observation accord with the known properties of other dehalogenases (Davies & Evans, 1962; carney *et al.*, 1964; Little & Williams, 1971; Goldman, 1972). Compared with other seudomonads, *P. putida* strain P3 showed significant differences in the range of substrates ydrolysed and the relative enzyme activities. For example, extracts of *Pseudomonas dehalo-ens* NCIB 9062 (strain K of Jensen, 1960) showed activity mainly towards MCA and to CA at 17% of the MCA rate. There was no activity in crude extracts of organisms grown n MCA against TCA, 2MCPA, 22DCPA or 3MCPA (Davies & Evans, 1962). Thus, the elative DCA rate was considerably lower than the DCA rate in *P. putida* strain P3 and iffered significantly in its response to chlorinated propionates. *Pseudomonas dehalogens* NCIB 9061 (strain R of Jensen, 1960) showed activity towards MCA and DCA, as well as MCPA and 22DCPA, at 35 and 9%, respectively, of the MCA rate (Little & Williams, 1971). Here too, in relation to the MCA rate, the activities towards chlorinated propionic acids were lower than in *P. putida* strain P3 and, furthermore, the 2MCPA:22DCPA activity ratio was greater than in *P. putida* strain P3. The apparent $K_m$ values for the four major substrates in *P. dehalogens* NCIB 9061 were at least an order of magnitude lower than those for same substrates in *P. putida* strain P3. These comparisons strongly suggest that there were major differences in the types of dehalogenases found in different pseudomonads.

The evidence presented in this paper also suggests that *P. putida* strain P3 contained at least two different dehalogenase proteins, one mainly involved in the dehalogenation of chlorinated acetic acids and the other mainly active against the chlorinated propionic acids. Firstly, two characteristically different pH profiles for each pair of substrates, together with the different dilution rate profiles in chemostat culture, provided some support for this suggestion. Secondly, the marked variations in the relative enzyme activities, particularly between the two groups of substrates, could have been due to variations in the relative proportions of two proteins, depending on growth conditions. The remarkably consistent 2:1 ratio for the 2MCPA:22DCPA activities in organisms grown in both closed and open culture indicated that these activities were common to a single dehalogenase. Thirdly, the thermal denaturation experiments with freshly prepared crude extracts showed a significantly different pattern for the loss of DCA activity which was more rapid than the loss of the 2MCPA and 22DCPA activities. These results indicated that the DCA activity was associated with a different protein from the 2MCPA and 22DCPA activities and, moreover, the parallel loss of activity against chlorinated propionates further indicated an association of these two activities. In addition, much of the DCA and MCA activity was cold labile and lost upon storage at 4 °C for 48 h. In these stored extracts the residual DCA activity seemed to be associated with the 2MCPA and 22DCPA dehalogenase activity. The loss of MCA activity was ambiguous, principally because its thermal denaturation curve followed that for 2MCPA and 22DCPA activity loss in freshly prepared extracts. Although MCA activity was lost at 4 °C, the MCA denaturation curve in stored crude extracts was strikingly different. We have recently purified the dehalogenases from *P. putida* strain P3 (Weightman *et al.*, 1979) and shown that there are two enzymes. One has activity towards 2MCPA and 22DCPA principally but with significant MCA activity and slight DCA activity. The second has major activity against DCA, significant activity against MCA but no activity towards 2MCPA and 22DCPA. These observations are entirely consistent with the results reported here and may serve to explain the somewhat anomalous behaviour for the MCA dehalogenase activity because it is associated with both proteins. There has been one previous demonstration that an organism contained two different dehalogenases (Goldman *et al.*, 1968) but each was separately induced, one by DCA and the other by MCA.

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